Ameliorative effect of Gamma-Oryzanol and *Morinda Citrifolia* in Polycystic Ovary Syndrome using animal model

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DECLARATION

I, hereby declare that the presented work in the thesis entitled "Ameliorative effect of Gamma-oryzanol and *Morinda citrifolia* in Polycystic Ovary Syndrome using animal model" in fulfilment of degree of Doctor of Philosophy (Ph.D.) is outcome of research work carried out by me under the supervision of Dr. Meenu working as Assistant Professor, in the Department of Pharmacology, School of Pharmaceutical Sciences of Lovely Professional University, Punjab, India. and Dr. Navneet Khurana, working as Professor, in the Department of Pharmacology, School of Pharmaceutical Sciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of the investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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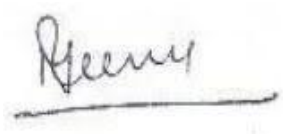
CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Ameliorative effect of Gamma-Oryzanol and *Morinda Citrifolia* in Polycystic Ovary Syndrome using animal model" submitted in fulfilment of the requirement for the award of degree of Doctor of Philosophy (Ph.D.) in the Department of Pharmacology, School of Pharmaceutical Sciences, is a research work carried out by Sayantika Chakraborty, 41800058, is Bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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Errors or inadequacies are all mine

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23-May-2025

DEDICATED TO

The liberation of Womanhood

ABSTRACT

Background and Purpose

Polycystic Ovary Syndrome (PCOS) is a heterogeneous disorder. Menstrual disorders, multiple cysts in ovaries, androgen and testosterone levels elevation, and obesity and insulin resistance kind of metabolic disorders are the common syndromes of PCOS. The massive increasing number of PCOS patients globally indicates the need for a more experimental approach to diminish the pathological conditions. Presently available management strategies are not up to the mark as they are found to provide only symptomatic relief. So, it is necessary to implement more experimental strategies to combat this disorder. Gamma oryzanol and *Morinda citrifolia*, these dietary components, found to be beneficial for the redaction of insulin resistance, helpful for the management of weight, effective in decreasing the oxidative stress and can regulate elevated oestrogen and testosterone level which are the main pathophysiological parameters of PCOS which indicates that there may be a causal relationship of Gamma oryzanol and *Morinda citrifolia*, with PCOS.

Experimental Approach

Sprague Dawley rats (87 Female + 10 Male), weighing 200-250 gm were used in the present experiment. Treatment was given to only the female rats. 87 Rats were grouped into 13 treatment groups, 6-7 animals per groups. 4 mg/kg estradiol valerate was injected to develop PCOS, through I.M. route and observed *the induction* of PCOS after 28 days. Clomifene citrate was nominated as a standard drug as it is widely used in PCOS patients. Gamma oryzanol and *Morinda citrifolia*, was administered afterwards to observe the combination effect of Gamma oryzanol and *Morinda citrifolia* with standard drug clomifene citrate. PCOS parameters are evaluated with histopathological, biochemical analysis along with estrous cyclicity, fertility study and statistical analysis.

Results

We observed that, gamma oryzanol and *Morinda citrifolia* both significantly decrease oestrogen and testosterone levels, helps in significant redaction of oxidative stress, promote the lessening of body weight as well as ovarian weight, also both of them have the potential to inverse the irregular phase pattern in estrous cycle and thus all of them accumulated the increment of fertilisation. On the All these effects are more significant while gamma oryzanol and *Morinda citrifolia* both are

associated with clomifene citrate treatment. In this study we tried combination of test drugs with themselves and also with standard drug individually and in a combination of all three. We used clomifene citrate as a test drug which is a renowned drug used to treat patient suffering with PCOS. In this study the combinations were created with 1. gamma oryzanol with clomifene citrate, 2. *Morinda citrifolia* with clomifene citrate, 3. gamma oryzanol with *Morinda citrifolia* and finally 4. gamma oryzanol, *Morinda citrifolia* with clomifene citrate. Gamma oryzanol with clomifene citrate shows a synergistic effect in comparison with the gamma oryzanol only treated group and shows additive effect in comparison with clomifene citrate only treated group. *Morinda citrifolia* with clomifene citrate shows a synergistic effect in comparison with the *Morinda citrifolia* only treated group and shows additive effect in comparison with clomifene citrate only treated group. The combination of two test drugs gamma oryzanol with *Morinda citrifolia* shows a synergistic effect in comparison with the individual treatment groups. The combination of gamma-oryzanol and Morinda citrifolia with clomifene citrate shows an additive effect compared to its combination with either the standard drug or the test drug

Conclusion

This finding indicates that gamma oryzanol and *Morinda citrifolia* together can produce a therapeutic benefit in PCOS patients. Further research is needed to explore the therapeutic potential of gamma-oryzanol and Morinda citrifolia in the treatment of PCOS. Future studies should focus on elucidating the underlying molecular pathways, including their roles in hormonal regulation, insulin sensitivity, inflammation, and oxidative stress. Additionally, validation of relevant biomarkers will be crucial to assess treatment efficacy and disease modulation. These investigations will provide deeper insights into their mechanisms of action and support the development of targeted interventions for PCOS.

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ABBREVIATION	FULL FORM
PCOS	Polycystic Ovary Syndrome
GO	Gamma-oryzanol
MC	*Morinda citrifolia*
p.o.	Per oral
CAT	Catalase
SOD	Superoxide dismutase
GST	Glutathione-S-transferase
P	Proestrus
O	Estrous
M	Metestrous
D	Diestrous
NaCl	Sodium chloride

Polycystic Ovary Syndrome widely popular as "PCOS" was first time identified in the year of 1935. Stein and Leventhal were the first to identify a consistent pattern of clinical symptoms affecting women of reproductive age. They observed a group of seven women presenting with ovarian enlargement featuring multiple cysts, accompanied by symptoms such as hirsutism and amenorrhea. Based on these findings, they collectively defined the condition as polycystic ovary syndrome (PCOS). Today, PCOS is a well-recognized endocrine disorder characterized by polycystic ovarian morphology, hyperandrogenism, and impaired oogenesis. (Pate, 2014).

In the USA, PCOS is the major popular endocrinological issue found in women population. In India, the reported PCOS cases is almost about 9.13% for the teenage patients; generally, the 15-18 age bracket (Nidhi et al., 2011).

Menstrual disorders, multiple cysts in ovaries, androgen and testosterone levels elevation, and obesity and insulin resistance kind of metabolic disorders are the common syndromes of PCOS. (Teede et al., 2010).

Associated pathophysiology regarding PCOS is still unclear because this disorder is a compilation of more than one clinical sign and symptoms. The elevation of Anti-Mullerian Hormone (AMH), disruption of Hypothalamus-Hypophysis Ovary Axis (HHOA), autosomal dominant genetic mutation are thought to be leads PCOS like clinical conditions (Sirmans & Pate, 2013).

Environmental toxins can provoke the pathogenesis of PCOS (Rutkowska & Diamanti-Kandarakis, 2016). Xenoestrogens like Bisphenol (BPA) is one of the popular environmental toxins found to be responsible for the manifestation of PCOS. Down the line, dichlorobiphenyl trichloroethane (DDT), polychlorinated biphenyls (PCB), polybrominated biphenyls (PBB), dioxins, methoxychlor (MCX) are also initiate PCOS (E. & E., 2013)**.**

Dietary supplements play a significant role in the management of various diseases, particularly metabolic disorders. In such conditions, a combination of dietary modifications, lifestyle changes, supplementation, and regular physical activity has been shown to alleviate symptoms and, in some cases, influence the underlying pathophysiology of the disease. For example, in individuals with diabetes mellitus, maintaining a balanced diet and achieving a healthy body weight can reduce or even eliminate the need for daily insulin injections, thereby enhancing overall quality of life.

Similar to diabetes mellitus, polycystic ovary syndrome (PCOS) is a metabolic disorder that can significantly impair a patient's quality of life. One of the most prominent challenges faced by individuals with PCOS is menstrual irregularity, which disrupts daily life and reproductive health. Elevated levels of androgens contribute to symptoms such as acne and hirsutism, which can negatively affect self-confidence and body image. In many cases, PCOS leads to infertility, further impacting a woman's self-esteem and emotional well-being. The consequences of PCOS extend beyond physical health, affecting mental, emotional, and social aspects of life.

Given the crucial role dietary interventions play in the management of metabolic disorders, this study focuses on two potential therapeutic candidates: gamma oryzanol and *Morinda citrifolia*. We aim to investigate their individual and combined effects on PCOS. Furthermore, the study will evaluate the synergistic potential of these compounds when administered alongside the standard pharmaceutical treatment, Clomifene citrate.

2.1. PCOS

Polycystic Ovary Syndrome widely popular as "PCOS" was first time identified in the year of 1935. Stein and Leventhal, the two scientists found a common pattern of a cluster of some disorder in women within their reproductive age, hence they nominated seven women, who were facing some clinical conditions like, engorgement of ovaries alongside several cysts, along with that, hirsutism and amenorrhea, Stein and Leventhal collectively termed these conditions as PCOS. Currently PCOS is a familiar disorder, illustrated by polycystic ovary, hyperandrogenism and disruption of oogenesis (Pate, 2014). Worldwide, on the basis of diagnosis criteria, 9 to 18% of women are suffering by PCOS throughout their reproductive age (Paixão et al., 2017). The clinical manifestations of hyperandrogenism, which is a cluster of various syndromes, leads to one of the most frightening causes of morbidities. Around 48.5 million women within 20 to 44 years old, are being diagnosed with PCOS, but these are only 6 to 15% of cases, while, 70% of women suffering with PCOS are still remains undiagnosed. Unclear pathophysiology and other pathogenic conditions like congenital adrenal hyperplasia, ovarian and adrenal neoplasms, obesity, Cushing's syndrome etc, generate complicacy in the diagnosis of this syndrome. The indications of PCOS cannot be restricted to the gynaecological domain because, it has been seen that, along with PCOS, prevalence of several other disorders like obesity, type 2 diabetes mellitus (DM2), dyslipidaemia, metabolic syndrome (MS), hypertension is elevated. Clinically, the onset of PCOS can arises earlier or throughout puberty. Clinical investigations revealed that, PCOS patients experience increased surge of gonadotropin releasing hormone (GnRH) which leads to increased secretion of luteinizing hormone (LH). This in turn, prematurely procures the excessive expression of LH receptors within developing ovarian follicles in early stages, leads to amplification of androgen production in ovary through which antral follicular development is arrested. This hypothesis is confirmed by the follicle arrest which is also observed in the ultrasound inspection of PCOS patients. Additionally, the ovaries of PCOS affected women also consist of higher quantity of maturing antral and preantral follicles that in turn causes the expansion of antrum and augmented granulosa cell erosion, furthermore enlargement of cystic follicles. The walls of granulosa cell become thinner with a thicker layer of theca cells on the surroundings of it (Rojas et al., 2014).

2.2. EPIDEMIOLOGY

In the USA, PCOS is the major popular endocrinological issue found in the women, which has the pervasiveness of 4% to 12% (Azziz et al., 2004; Knochenhauer et al., 1998)**.** (Azziz et al., 2004; Knochenhauer et al., 1998)**.** PCOS is generally found in almost 10% of woman while they undergoing any kind of gynaecological screening throughout their reproductive age. (Cahill, 2009)**.** The worldwide prevalence of PCOS is approximately 9.2% (95% CI: 6.8–12.5%). Estimates differ based on diagnostic standards: 5.5% (95% CI: 3.9–7.7%) using NIH guidelines, 11.5% (95% CI: 6.6–19.4%) according to Rotterdam criteria, and 7.1% (95% CI: 2.3–20.2%) with AES criteria. When based on self-reported data, the prevalence is around 11% (95% CI: 5.2–21.8%) (Salari et al., 2024).

In India, the reported PCOS cases is almost about 9.13% for the teenage patients; generally, the 15-18 age bracket. It was found with a study conducted in South India with 460 teenage girls. Through USG (Ultrasonography) 9.13% participants shows multiple cystic structure in their ovaries, in these 9.13% participants, 30.95% having hyperandrogenism, 97.92% having oligomenorrhea (Nidhi et al., 2011)**.**

2.3. SIGNS AND SYMPTOMS

Menstrual disorders: Most popular clinical feature of PCOS is either oligomenorrhea or amenorrhea. Along with them, different types of menstrual complications may also happen(Azziz et al., 2016) . Oligomenorrhea refers to infrequent menstrual periods, typically defined as having fewer than six to eight menstrual cycles per year. In PCOS, oligomenorrhea is often due to absence of ovulation process termed as anovulation, where the regulation of egg release by ovaries are distorted, leading to disrupted hormonal signaling and irregular shedding of the uterine lining (Norman et al., 2007). Amenorrhea is the absence of menstrual periods for three consecutive cycles or more. It can be classified as primary (never having menstruated) or secondary (the cessation of periods after previously normal cycles) (Fauser et al., 2012). This condition is often a result of prolonged anovulation and is common in women with PCOS. Menorrhagia refers to heavy or prolonged menstrual bleeding. Women may experience periods that last longer than seven days or have excessive blood flow (Mentula et al., 2011). In PCOS, irregular hormone levels can lead to

endometrial hyperplasia (thickening of the uterine lining), resulting in heavy bleeding when menstruation occurs. Menstrual cycles may vary significantly in length, often ranging from very short to very long intervals (more than 35 days). Women may experience unpredictable timing of menstruation, making it difficult to anticipate periods and manage associated symptoms (Goodman et al., 2015).

Infertility: Chronic anovulation give birth to infertility. Anovulation or irregular ovulation along with alteration in the levels of gonadotropins, insulin etc. in PCOS patients can leads to infertility. Polycystic Ovary Syndrome (PCOS) is a widespread and fairly common endocrine disorder, which has a substantial and often profound impact on reproductive health, influencing various aspects of hormonal balance and fertility, with infertility being one of its most concerning complications. Understanding the mechanisms, implications, and management strategies for infertility in PCOS is crucial for affected individuals. Along with chronic anovulation, hormonal imbalance like elevated levels of androgens (male hormones) and an imbalance in luteinizing hormone (LH) and follicle-stimulating hormone (FSH). This disruption inhibits the normal ovarian function required for ovulation. Many women who are diagnosed with PCOS often experience irregular or even absent menstrual cycles, which can make it challenging to predict ovulation accurately, complicating the process of conception and fertility tracking. Irregular cycles can hinder natural conception (Hussein et al., 2024). Insulin resistance is common in PCOS and can lead to higher insulin levels, which may stimulate the ovaries to produce more androgens. This exacerbates anovulation and may affect egg quality. In PCOS, multiple immature follicles may develop, but they often fail to mature and release an egg. Instead, these follicles can become cysts, contributing to the polycystic appearance of the ovaries (Diamanti-Kandarakis & Dunaif, 2012).

Psychological and Emotional Impact of infertility leads to Stress and Anxiety and Body Image Issues as the challenges of conceiving can lead to heightened stress and anxiety, impacting overall mental health and physical symptoms associated with PCOS, such as hirsutism and weight gain, can affect self-esteem and body image, further complicating emotional well-being (Tan et al., 2008). In the treatment line, lifestyle modifications like weight management, diet and exercise play a significant role. In the studies it found that, even a modest weight loss of 5-10% can improve insulin sensitivity and restore ovulation in many women. A balanced diet and regular physical activity are crucial in managing symptoms and improving fertility. In medications, Clomiphene

Citrate, Letrozole and Metformin are some best options available (Attia et al., 2023; Guang et al., 2018; Takasaki et al., 2018). Clomiphene Citrate is marketed as an oral medication is often the first-line treatment for inducing ovulation. It works by stimulating the hypothalamus to increase the release of hormones that promote ovulation (Takasaki et al., 2018). Letrozole is an alternative to clomiphene, letrozole has been shown to be effective in inducing ovulation and is sometimes preferred, especially in women who are resistant to clomiphene (Bansal et al., 2021). Metformin improves insulin sensitivity and can help restore regular ovulation in women with PCOS for overweight population. Assisted Reproductive Technologies (ART) are now a days used to overcome infertility. Intrauterine Insemination (IUI) and In Vitro Fertilization (IVF) are widely acceptable technologies (Sirmans & Pate, 2013). Intrauterine Insemination helps in fertilization by insertion of sperm inside uterus during the ovulation phases. IVF is the invitro fertilization a fertility treatment that helps people have children by combining eggs and sperm in a laboratory and then transferring the fertilized eggs to the uterus.

High levels of masculinizing hormones: Elevated levels of androgen is another common pathological condition in PCOS which is termed as Hyperandrogenism. Hypermenorrhoea, acne and either hirsutism or androgenic alopecia are the consequences of hyperandrogenaemia. The main hormones involved include testosterone, dehydroepiandrosterone (DHEA), and androstenedione. Understanding these hormones, their sources, and the implications of their imbalances is crucial for managing PCOS effectively.

Testosterone: Testosterone is considered the primary male sex hormone, playing a significant role in the development of male characteristics, but it is also naturally present in females, though in much smaller amounts compared to males(Burger, 2002). t plays a vital and indispensable role in numerous key bodily functions, such as the regulation of menstrual cycles, the upkeep of bone density, as well as the support of muscle mass and strength (Braunstein, 2002). In women, testosterone is produced in ovaries and adrenal glands. Ovarian theca cells produce androgens, which include testosterone. The adrenal cortex produces small amounts of androgens (Goodman et al., 2015). Women with PCOS often have elevated total and free testosterone levels. High levels of free testosterone are particularly concerning as they are the active form that affects tissues (Azziz et al., 2004). Increased hair growth in male-pattern areas (face, chest, back) due to heightened sensitivity of hair follicles to testosterone causes hirsutism. Increased sebum production can lead to

acne, a common symptom in PCOS(Fauser et al., 2012). Thinning of hair or male-pattern baldness occurs because of the effects of elevated testosterone on hair follicles. Blood tests measure total testosterone levels(Sirmans & Pate, 2013). Normal ranges for women typically fall between 15-70 ng/dL, but levels in women with PCOS often exceed this range (Azziz et al., 2004).

Dehydroepiandrosterone (DHEA): DHEA is an androgen produced primarily by the adrenal glands. It serves as a precursor for other sex hormones, including testosterone and estrogen. DHEA is synthesized in the adrenal cortex and is released into the bloodstream, where it can be converted to other hormones in various tissues. Women with PCOS often exhibit higher levels of DHEA and its sulfate form (DHEAS) (Azziz et al., 2004). Elevated DHEA levels may increase testosterone production, worsen hyperandrogenism symptoms like hirsutism, and disrupt the menstrual cycle, contributing to anovulation. Blood tests can assess DHEA and DHEAS, which typically range from 35–430 µg/dL in women; higher levels are common in PCOS (Sirmans & Pate, 2013) (Fritz & Speroff, 2011).

Androstenedione: Androstenedione is another androgen that serves as a precursor to both testosterone and estrogen (Labrie et al., 1997). It is produced in the ovaries, adrenal glands, and peripheral tissues (Burger, 2002). Theca cells and adrenal glands both produce androstenedione. In PCOS, elevated androstenedione increases testosterone levels, worsening hyperandrogenism symptoms (Azziz et al., 2004). Similar to other androgens, elevated androstenedione can disrupt normal menstrual cycles (Fauser et al., 2012). Increased levels can contribute to the development of excessive hair growth i.e., hirsutism and acne (Goodman et al., 2015). Blood tests typically measure androstenedione levels, which should normally fall between 0.5-3.0 ng/mL for women (Fritz & Speroff, 2011). Elevated levels are frequently found in PCOS(Sirmans & Pate, 2013).

Hormonal Interactions and Implications

- **Insulin Resistance**: Many women with PCOS also exhibit insulin resistance, which can lead to elevated insulin levels. High insulin levels stimulate the ovaries to produce more androgens, compounding the effects of testosterone, DHEA, and androstenedione (Diamanti-Kandarakis & Dunaif, 2012). This relationship forms a vicious cycle, where increased androgen levels further exacerbate insulin resistance (Legro et al., 1999).

- **Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) Ratio**: Women with PCOS typically have an increased LH-to-FSH ratio (often > 2:1). Elevated LH levels stimulate the production of androgens in the ovaries, while low FSH levels impair follicular development and ovulation (Franks, 1995).

Elevated levels of masculinizing hormones in PCOS-testosterone, DHEA, and androstenedione-play a significant role in the clinical manifestations of the syndrome, including menstrual irregularities, infertility, and symptoms of hyperandrogenism (Azziz et al., 2004). Understanding these hormones and their interrelations is essential for effective diagnosis and treatment, allowing for improved quality of life and reproductive health in women with PCOS (Goodman et al., 2015).

Metabolic syndrome: Insulin resistance, elevated level of homocysteine are the other metabolic complications of PCOS. These clinical observations of PCOS leads to obesity like metabolic syndrome (Teede et al., 2010). Polycystic Ovary Syndrome (PCOS) is a complex endocrine disorder that influences multiple aspects of health, not only impairing reproductive health but also significantly heightening the likelihood of developing metabolic syndrome. This metabolic condition is characterized by a combination of risk factors, including high blood pressure, elevated blood sugar levels, abnormal cholesterol levels, and excess abdominal fat (Essah & Nestler, 2006). When these factors occur together, they substantially increase the risk of developing serious health problems, such as cardiovascular disease, stroke, and type 2 diabetes. Understanding the relationship between PCOS and metabolic syndrome is crucial for effective management and prevention of associated health complications, including:

- **Obesity**: Increased waist circumference indicating visceral fat accumulation.
- **Insulin Resistance**: Reduced sensitivity to insulin, leading to higher blood glucose levels.
- **Dyslipidaemia**: Abnormal lipid levels, often characterized by elevated triglycerides and reduced HDL (high-density lipoprotein) cholesterol.
- **Hypertension**: Elevated blood pressure.
- **Proinflammatory State**: Increased levels of inflammatory markers, which can contribute to cardiovascular risk. (Chen et al., 2021)

In order to receive a diagnosis of metabolic syndrome, an individual generally needs to meet at least three of the following key criteria:

1. **Abdominal obesity**, characterized by a waist circumference greater than 40 inches in men or 35 inches in women.
2. **Elevated triglyceride levels**, with a measurement of 150 mg/dL or higher.
3. **Low levels of HDL cholesterol**, specifically less than 40 mg/dL in men or under 50 mg/dL in women.
4. **High blood pressure**, with readings of 130/85 mmHg or greater.
5. **Increased fasting glucose**, with a level of 100 mg/dL or more (*Metabolic Syndrome - Symptoms & Causes - Mayo Clinic*, 2025).

These factors, when present together, significantly increase the risk of developing serious health conditions.

- Metabolic syndrome is notably more common among women with PCOS than in the general population. Research indicates that:(*Metabolic Syndrome - Symptoms & Causes - Mayo Clinic*, 2025)
- Around 30% to 70% of women with PCOS may meet the diagnostic criteria for metabolic syndrome, with the exact percentage varying based on the specific population studied and the diagnostic criteria applied.
- The prevalence is especially high among women with PCOS who are obese, with estimates suggesting that more than 80% of this group may be affected by metabolic syndrome.
- This elevated risk highlights the importance of closely monitoring metabolic health in women with PCOS, particularly those who are overweight or obese.

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Mechanisms Linking PCOS and Metabolic Syndrome

- **Insulin Resistance**: Insulin resistance is a hallmark of PCOS, plays a central role, and a primary driver of metabolic syndrome. It leads to compensatory hyperinsulinemia (high insulin levels), which can further exacerbate androgen production from the ovaries, creating a vicious cycle (Diamanti-Kandarakis et al., 2006). Elevated insulin levels contribute to weight gain, particularly visceral fat accumulation, which is linked to further metabolic disturbances (Teede et al., 2010).
- **Obesity**: A significant number of women with PCOS are either overweight or obese, which further complicates the management of the condition and increases the risk of developing

associated health issues, such as metabolic syndrome and cardiovascular disease, which is a significant risk factor for developing metabolic syndrome (Moran et al., 2011). Adiposity, especially visceral fat, is associated with insulin resistance and inflammation, contributing to the overall metabolic dysregulation (Sam, 2007).

- **Hormonal Imbalances**: Elevated levels of androgens (such as testosterone) in PCOS can influence body fat distribution, favoring abdominal fat accumulation, which is a key component of metabolic syndrome (Azziz et al., 2004; Barber et al., 2006; Sam, 2007).
- **Inflammation**: Women with PCOS often exhibit a chronic low-grade inflammatory state, characterized by increased levels of inflammatory markers (e.g., C-reactive protein). This inflammation can contribute to insulin resistance and cardiovascular risk (González et al., 2006; Tarkun et al., 2004).
- **Dyslipidemia**: PCOS is often associated with abnormal lipid profiles, including elevated triglycerides and lower HDL cholesterol. These changes are linked to insulin resistance and obesity, both of which are integral components of metabolic syndrome (LEGRO, 2004; Wild, 2012).

Clinical Implications of Metabolic Syndrome in PCOS

- **Increased Cardiovascular Risk**: Women with PCOS and metabolic syndrome have a heightened risk of cardiovascular diseases. This includes a greater likelihood of developing atherosclerosis, hypertension, and other cardiovascular complications (Zhang et al., 2020).
- **Type 2 Diabetes:** Insulin resistance in PCOS significantly increases the risk of developing type 2 diabetes. Studies indicate that women with PCOS are up to seven times more likely to develop diabetes compared to their peers without the syndrome (Anagnostis et al., 2021).
- **Endometrial Health:** Chronic anovulation and the resulting unopposed estrogen exposure increase the risk of endometrial hyperplasia and cancer in women with PCOS, particularly when compounded by metabolic syndrome (Johnson et al., 2023).
- **Mental Health:** The psychological impact of dealing with the physical symptoms of PCOS, coupled with the stress of managing metabolic syndrome, leads to anxiety as well as bipolar disorder, depression etc (Dewani et al., 2023).

2.4. PATHOPHYSIOLOGY

Alteration in various physiological events is happening throughout oogenesis and leads to the disruption of folliculogenesis for a PCOS patient. But no individual reason for PCOS has not been identified till now so the most recognized statement is a multi-factorial phenomenon, where interfaces between environmental toxins with intrinsic factors manifests the PCOS (Diamanti-Kandarakis & Dunaif, 2012; Rosenfield & Ehrmann, 2016).

The initiation of folliculogenesis is cooperated with elevated levels of AMH (Anti-Mullerian Hormone) which is comprises of 560 amino acid peptide belongs to TGF-β family. The granulosa cells secrets AMH and express in small antral follicles and powerfully inhibit the primordial follicle formations and also follicle sensitivity of follicles towards FSH (follicle-stimulating hormone) (Piltonen et al., 2023). AMH levels gradually reduction with the increment of follicular size. This attenuation of AMH is important for the progression of ovulation. Elevated levels of AMH is a hallmark of PCOS patients indicating the disruption of oogenesis as well as worsening fertility rate (Pigny et al., 2003).

Disruption of the feedback mechanism of HHOA (Hypothalamus-Hypophysis Ovary Axis) is also associate with the pathogenesis of PCOS. HHOA guides the secretion of GnRH (Gonadotropin Releasing Hormone) and LH (Luteinizing Hormone) (Lonardo et al., 2024). Disruption of HHOA results the elevation of the secretion of GnRH and LH, as a result, androgen synthesis increases as GnRH and LH induces androgen synthesis in ovarian theca cells. So, the disturbance of HHOA leads to hyperandrogenaemia, a common clinical feature of PCOS (Arao et al., 2019).

Exogenous stimulations like xenoestrogens and environmental disruptors found to be provoking the pathogenesis of PCOS. Genetic influences are also considered as one of the reasons for the pathogenesis of this syndrome. The most accepted theory is Mendelian pattern of inheritance, where PCOS is assumed to be an autosomal dominant disorder (Azziz et al., 2016). According to this hypothesis, hyperandrogenaemia caused by the mutations of the genes which express androgen receptor, steroidogenic enzymes and SHBG known as sex hormone binding globulin (Sirmans & Pate, 2013).

2.5. AETIOLOGY

Animal models are considered as a principal tool in the investigation of pathophysiology of diseases. Various animal studies were performed to get an idea about the exact pathophysiology and aetiology of PCOS. The administration of testosterone, oestrogen and environmental disruptors found to be established a PCOS like condition in various animal studies. (Paixão et al., 2017).

Androstenedione, Testosterone propionate + hCG, Testosterone + HFD, Testosterone propionate, DHEA, DHT, eCG (equine chorionic gonadotropin) + DHEA etc. androgenic drugs are used in the induction of PCOS (Divyashree et al., 2019; Osuka et al., 2018).

Oestrogenic compounds are also used to prompt a PCOS-like phenomenon in animals (van Houten & Visser, 2014). Oestrogen has an ability to prolong estrous cycle, as a result, oogenesis process is disrupted and follicular maturation is arrested. These conditions mimic the morphological characteristics of woman with PCOS. Estradiol valerate, Estradiol benzoate, Bisphenol A (BPA) and Estradiol-17β are common oestrogenic compound to induce PCOS

Investigational exposure of the endocrine disruptors causes the impairment of regular reproductive functions which in turn helps in the aggregation and development of PCOS like clinical features. A list of environmental toxins like, PCB (polychlorinated biphenyls), PBB (polybrominated biphenyls), dioxins, some of the pesticides and herbicides like DDT (dichlorobiphenyl trichloroethane) and MCX (methoxychlor), and fungicides like vinclozolin, heat stabilisers and chemical catalysts like tributyltin, plastic contaminants like BPA and plasticizers like phthalates had been reported for the development of PCOS (E. & E., 2013; Hewlett et al., 2016; Lindenau et al., 1994; Meserve & Cromwell, 2011; Morán et al., 2001; Tiemann, 2008; Uzumcu et al., 2006; Wu et al., 2006).

2.6. Gamma oryzanol

Gamma-oryzanol is group of lipids found in rice bran oil. Rice bran oil can be excreted out using a HPLC from the rice bran oil. To separate the individual components of gamma oryzanol a reverse-phase HPLC is found to be useful technique. (Xu & Godber, 1999a)

Gamma-oryzanol is traditionally used in countries like Japan and China to combat high cholesterol, stomach upset, mild anxiety and most significantly some menopausal symptoms. In USA it is used in sport industry as a supplementary (Eslami et al., 2014a).

Gamma oryzanol is a natural compound primarily extracted from rice bran oil. This complex mixture consists of ferulic acid esters and various phytosterols, making it a subject of interest in nutritional science and health supplements. Known for its wide range of potential health benefits, including antioxidant, cholesterol-lowering, and anti-inflammatory effects, gamma oryzanol has been studied for its role in cardiovascular health, athletic performance, and more. This document will explore the chemical properties, mechanisms of action, health benefits, applications, safety, and potential future research directions related to gamma oryzanol (*Gamma Oryzanol - an Overview | ScienceDirect Topics*, 2025).

Chemical Composition and Structure

Gamma oryzanol is not a single chemical entity but rather a complex mixture of several compounds. The principal constituents include:

- Ferulic Acid: A potent antioxidant that is an integral part of gamma oryzanol's structure.
- Phytosterols: These plant-derived compounds, such as campesterol, stigmasterol, and beta-sitosterol, contribute to its lipid-lowering properties.

The chemical structure of gamma oryzanol can vary based on its source and extraction method. The main structure involves ferulic acid esterified with sterols, giving it lipid-soluble characteristics that enhance bioavailability(Islam et al., 2014).

Extraction Process

Gamma oryzanol is typically obtained from rice bran oil through methods such as cold pressing, supercritical fluid extraction, or solvent extraction. The purity and concentration of gamma oryzanol can vary significantly based on the extraction technique used. Cold pressing is often preferred for retaining the bioactive compounds in their natural state (Baixinho et al., 2025).

Mechanisms of Action

Understanding how gamma oryzanol functions biologically is crucial for appreciating its health benefits. Key mechanisms include:

Antioxidant Activity:

Gamma oryzanol is a powerful antioxidant that helps neutralize free radicals, reducing oxidative stress. This is critical because oxidative stress is linked to the aging process and various diseases, including cancer and cardiovascular conditions (Islam et al., 2014).

Cholesterol Regulation:

Research indicates that gamma oryzanol may inhibit intestinal cholesterol absorption and promote bile acid excretion. By doing so, it can help lower total cholesterol levels, cholesterol. All these became a potential threat to cardiac incidents (Esperança et al., 2022).

Hormonal Modulation:

Gamma oryzanol is believed to modulate hormones such as testosterone and estrogen. This hormonal balance can have beneficial effects on muscle growth, recovery, and overall physical performance, particularly in athletes (Kuang et al., 2024).

Anti-inflammatory Properties:

By inhibiting pro-inflammatory cytokines and enzymes, gamma oryzanol may help reduce inflammation, which is implicated in various chronic diseases, including arthritis, diabetes, and cardiovascular issues (Panchal et al., 2017).

Impact on Neurotransmitters:

Some studies suggest that gamma oryzanol may influence the levels of certain neurotransmitters, including serotonin and dopamine, potentially affecting mood and stress levels (Mastinu et al., 2019).

Health Benefits

The potential health benefits of gamma oryzanol are extensive, making it a valuable compound for various applications:

Cardiovascular Health:

Extensive research has revealed that gamma oryzanol can improve lipid profiles by lowering LDL cholesterol and triglycerides while increasing HDL (high-density lipoprotein) cholesterol. This may contribute to a reduced risk of atherosclerosis and other cardiovascular diseases (Rao et al., 2016; Rong et al., 1997)

Support for Athletic Performance:

Gamma oryzanol is often marketed in sports supplements due to its purported effects on strength and endurance. Its role in enhancing testosterone levels may aid in muscle development and

recovery, making it popular among bodybuilders and athletes (Ahn et al., 2021; Eslami et al., 2014b).

Anti-Aging Effects:

As an antioxidant, gamma oryzanol can help protect skin cells from oxidative damage, potentially reducing the appearance of aging. It is sometimes included in skincare products for its protective benefits against UV radiation and environmental stressors (Rungratanawanich et al., 2018).

Weight Management:

Some evidence suggests that gamma oryzanol can aid in weight management by promoting fat metabolism and reducing fat accumulation. This may make it a useful component of weight loss supplements (Francisqueti et al., 2017; O. Wang et al., 2015).

Gastrointestinal Health:

Gamma oryzanol may support gut health by promoting the growth of beneficial gut bacteria. A healthy gut microbiome is essential for overall health and can influence everything from digestion to immune function (Trinovita et al., 2018).

Applications

Dietary Supplements:

Gamma oryzanol is widely available in dietary supplements, often aimed at athletes and individuals seeking to manage cholesterol levels. It is typically offered in capsule, tablet, or powder form (Eslami et al., 2014c).

Functional Foods:

As a functional ingredient, gamma oryzanol can be incorporated into various food products, such as health bars, cereals, and snacks, to enhance their nutritional profile (Gunathunga et al., 2024)

Cosmetic Products:

Due to its antioxidant properties, gamma oryzanol is increasingly used in cosmetic formulations. Environmental exposure related damages can be reversed and improve skin elasticity and hydration (Saikia & Dutta, 2022).

Pharmaceutical Research:

Emerging research is investigating gamma oryzanol's potential therapeutic effects in conditions like diabetes, hypertension, and neurological disorders. Its multifaceted actions may lead to novel treatment avenues (Rao et al., 2016; O. Wang et al., 2015).

Safety and Dosage

Gamma oryzanol is generally regarded as safe when consumed within recommended dosages. Typical doses range from 100 mg to 300 mg per day, although individual needs may vary based on health goals and conditions (*GAMMA-ORYZANOL: Overview, Uses, Side Effects, Precautions, Interactions, Dosing and Reviews*, 2024).

Potential Side Effects:

Adverse effects are rare but can include gastrointestinal discomfort, allergic reactions, or interactions with medications (Kahlon, 2009) It's essential for individuals, especially those with underlying health conditions or those taking medications, to consult a healthcare professional before starting supplementation.

As interest in natural health products grows, further research into gamma oryzanol is warranted. Areas for future exploration include:

- Long-term Effects
- Mechanistic Studies: Investigating the specific biochemical pathways through which gamma oryzanol exerts its effects can provide deeper insights into its potential therapeutic applications (Wilson et al., 2007) .
- Comparative Studies: Research comparing gamma oryzanol with other natural compounds may help establish its efficacy in various health domains (Xu & Godber, 1999b).

Gamma oryzanol is a promising natural compound with a range of potential health benefits, including antioxidant, cholesterol-lowering, and anti-inflammatory effects (Xu & Godber, 1999b). Its multifaceted properties make it a valuable addition to dietary supplements and functional foods. As research continues to evolve, gamma oryzanol may play an increasingly important role in promoting health and wellness, paving the way for innovative applications in both nutritional science and medicine. With its rich history and potential future benefits, gamma oryzanol stands out as a remarkable compound worthy of further investigation.

Rationale to consider Gamma oryzanol for PCOS

Form studies it is observed that, Gamma oryzanol increase the level of estrogen by modifying gene expression. Gamma oryzanol is observed to be increase the expression of CaBP9K and C3 gene.

CaBP9K gene: In the uterus the progesterone usually modifies the responses stimulated by estrogen. To examine the interactions within estradiol and progesterone, CaBP9k (Calbindin-D

9k) which is a 17 beta-estradiol-responsive gene (found in uterus) is used as a marker (L'Horset et al., 1993).

The 9KDa (a calcium-binding protein which is dependent on vitamin D), also termed as calbindin-D9K is present in the placenta and uterus (in epithelial cells). Before the start of labor, if myometrial tissues are examined, then higher levels of CaBP9K can be found (Miller et al., 1994). During gestation period the levels of estrogen increase significantly and the peak level touches during the 3rd trimester (R. Sarkar et al., 2014). From these information we can conclude that. The increase CaBP9K expression can increase the level of estrogen.

C3 gene: C3 gene (complement C3) helps in the activation of the complement cascade (or complement system which belongs to the immune system (non-specific). The activated complement cascade increases the potential of antibodies as well as the phagocytic cells to excreted out the damaged cells. Also, this cascade helps to endorse inflammation (Charles A Janeway et al., 2001). C3 gene can only be found in the epithelial cells of uterus and its promotor is highly responsive to estrogen stimulations. Hence C3 gene is used as a marker to track the steroidal activities in uterus (Gilbert, 2000) (Sundstrom et al., 1989). All these information signifies the relation of estrogen with the C3 gene expression.

Role of Gamma oryzanol in Upregulate C3 gene and Increase expression of CaBP9K:

To understand the regulations of C3 and CaBP9K in the uterus and also identify the molecular mechanisms, preclinical study was conducted with ovariectomized rats. These rats were treated with the bioactivates of brown rice (rich in gamma oryzanol) and evaluated to estrogen or remifemin.

The results showed that the up regulation of C3, IL-4, ER- β is present with the group treated with estrogen for obvious reasons. With 200mg/kg gamma oryzanol the up regulations observed for CaBP9k, ER- β genes. The level of FSH and estrogen also raised with the 200mg/kg gamma oryzanol treated group.

As a conclusion of this study, we can conclude that, gamma oryzanol have an up regulatory effect on the uterine genes (Muhammad et al., 2013).

Role of Gamma oryzanol FSH:

Follicle stimulating hormone (FSH) is play a significant role in the maturation of ovarian follicles. The GNRH (gonadotropin-releasing hormone) helps to produce FSH by anterior pituitary by receiving the stimulation from Hypothalamus.

In preclinical studies it is observed that FSH level can increase with Gamma oryzanol. For rat model after the treatment of 8 weeks the FSH level is found to be significantly increased (Muhammad et al., 2013).

Hence from studies we observed that, gamma oryzanol can increase the level of estrogen through upregulate C3 gene and increase the expression of CaBP9K. Gamma oryzanol can also increase the serum oestrogen level. Along with this Serum FSH level is also increase after a continuous exposure of gamma oryzanol. Increase level of oestrogen and increase serum FSH directly linked to a positive folliculogenesis. We know that, in PCOS the folliculogenesis is disrupted at many levels, so hence we can impose the fact that gamma oryzanol can promote a positive folliculogenesis in the condition of PCOS.

2.7. *Morinda citrifolia*

Morinda citrifolia also known as Noni is a plant mainly found in south Asian countries as well in Australia. Traditionally Noni has been found widely used in treatment of cancer, gastric ulcers, depression and also to increase physical heath as a health drink. Noni is basically a fruity plant from family Rubiaceae, this noni fruit is used as a famine food. In the region of pacific island noni is used as a staple food, eaten cooked as well as raw as a fruit. In Austronesia noni is used as a dye to make the “Batik” designs (Hou et al., 2025).

Morinda citrifolia, commonly known as noni, is a tropical fruit-bearing tree native to Southeast Asia and Australasia. It belongs to the Rubiaceae family and has been utilized for centuries in traditional medicine for its wide array of health benefits. The fruit, leaves, and other parts of the plant are rich in bioactive compounds, making *Morinda citrifolia* a subject of increasing interest in the fields of nutrition, herbal medicine, and pharmacology. This overview delves into the plant’s botanical characteristics, chemical composition, health benefits, traditional uses, applications, and safety considerations (B. Sarkar et al., 2022).

Botanical Characteristics

Taxonomy and Description

Morinda citrifolia is a flowering plant that can grow up to 10 meters tall. The tree features large, glossy leaves that are elliptical to ovate in shape, typically measuring between 10 to 30 centimetres in length (McClatchey, 2003). The flowers are small, tubular, and usually white to pale yellow, forming in clusters. The fruit is oval-shaped, measuring about 7 to 10 centimetres long, and ripens to a yellowish or white colour. The fruit has a distinctive smell and taste that is often described as strong and pungent (Samoylenko et al., 2006).

Habitat

This hardy plant thrives in tropical and subtropical climates, preferring well-drained soils and areas with full sun exposure. It is often found in coastal regions, forest margins, and even in disturbed areas, showcasing its resilience and adaptability (Gupta & Singh, 2013a).

Chemical Composition

Morinda citrifolia is rich in a variety of bioactive compounds, contributing to its medicinal properties. Key constituents include:

Anthraquinones: These compounds, including morindone and alizarin, have been studied for their potential anti-inflammatory and analgesic effects(Potterat & Hamburger, 2007) .

Polysaccharides: The presence of complex carbohydrates may enhance immune function and promote gut health (Palu et al., 2007).

Flavonoids: These antioxidants play a role in reducing oxidative stress and inflammation (M. Y. Wang et al., 2002).

Vitamin C: Noni fruit is a significant source of vitamin C, which is essential for immune function and skin health (Boonanantanasarn et al., 2014).

Minerals: Noni contains important minerals such as potassium, calcium, and magnesium, which are vital for various bodily functions (Boonanantanasarn et al., 2014).

Nutritional Profile

The fruit of *Morinda citrifolia* believing of having various health benefits as it having a higher content of minerals, lesser calories and more vitamins. Its juice is often consumed for its health benefits, while the fruit can also be eaten raw or processed into various products (Kim et al., 2020).

Health Benefits

Morinda citrifolia has been attributed with numerous health benefits, supported by both traditional use and emerging scientific research:

Immune Support:

Noni is believed to enhance immune function due to its high vitamin C content and the presence of polysaccharides. Studies have suggested that noni juice may stimulate immune responses, making it beneficial in preventing infections (Tanikawa et al., 2021).

Anti-inflammatory Effects:

The anthraquinones and flavonoids in noni have shown anti-inflammatory properties in various studies. These compounds may help alleviate conditions such as arthritis and other inflammatory disorders (Tanikawa et al., 2021).

Antioxidant Properties:

Noni is rich in antioxidants, which help combat oxidative stress, a contributor to chronic diseases like cancer and heart disease. The flavonoids and vitamin C work synergistically to protect cells from damage (Basar et al., 2010)

Pain Relief:

Traditional uses of *Morinda citrifolia* include its application as a natural analgesic. Some studies indicate that its constituents may help reduce pain and improve overall well-being (Basar et al., 2010) .

Digestive Health:

The polysaccharides found in noni may promote gut health by supporting the growth of beneficial bacteria. Noni is also believed to help with digestive issues such as constipation and bloating (Do et al., 2022).

Metabolic Health:

Research has indicated that *Morinda citrifolia* may help regulate blood sugar levels and improve lipid profiles, making it a candidate for managing diabetes and cardiovascular health (Inada et al., 2017).

Skin Health:

Topical applications of noni extracts are used in traditional medicine for skin conditions, including eczema and psoriasis. The antioxidant and anti-inflammatory properties may aid in skin healing and rejuvenation (Tanikawa et al., 2021; Zhou & Huang, 2022).

Traditional Uses

Morinda citrifolia has a long history of use in traditional medicine across various cultures. In Polynesian medicine, noni has been used for centuries to treat ailments ranging from infections to

digestive issues. Traditional healers utilize different parts of the plant, including the fruit, leaves, and roots, for their therapeutic properties.

Traditional Remedies:

The fruit is often consumed as juice or in dried form to enhance overall health. The leaves are sometimes used in poultices for wound healing and skin conditions (McClatchey, 2003).

Cultural Significance:

In many Pacific Island cultures, noni is considered a sacred plant, symbolizing health and well-being. It is often included in traditional rituals and ceremonies (Torres et al., 2017).

Applications

Dietary Supplements:

Noni juice and extracts are widely marketed as dietary supplements, often promoted for their health benefits. They are available in various forms, including capsules, powders, and liquid extracts (Kharaeva et al., 2022; Philips & Theruvath, 2024).

Functional Foods:

Due to its nutritional profile, noni is incorporated into health foods and beverages. Products like noni juice blends and smoothies are popular for their purported health benefits .

Cosmetics and Skincare:

The antioxidant and anti-inflammatory properties of noni make it a valuable ingredient in cosmetic formulations. It is used in creams, lotions, and serums designed to promote skin health (Jugreet et al., 2022).

Pharmaceutical Research:

Ongoing research is investigating the potential therapeutic applications of *Morinda citrifolia* in managing various health conditions, including cancer, diabetes, and inflammatory diseases (Torres et al., 2017).

Safety and Dosage

Morinda citrifolia is generally considered safe for most individuals when consumed in moderation. However, some considerations include:

- **Potential Side Effects:**

While side effects are rare, some individuals may experience gastrointestinal discomfort or allergic reactions. It's essential to consult a healthcare provider before starting any new supplement,

especially for those with pre-existing health conditions or those taking medications (Posadzki et al., 2013).

- **Dosage Recommendations:**

There is no standardized dosage for noni products, but typical recommendations range from 30 to 120 ml of noni juice per day. Individuals should follow product instructions and consult healthcare professionals for personalized advice (Nima et al., 2012).

As interest in *Morinda citrifolia* continues to grow, further research is needed to explore its full potential:

- **Clinical Trials:**

To validate the health claims associated with noni and determine effective dosages for various health conditions there should be a good number of clinical trials conducted (Abou Assi et al., 2017).

- **Mechanistic Studies:**

Understanding the biochemical mechanisms by which *Morinda citrifolia* exerts its effects can provide insights into its therapeutic applications and potential synergies with other compounds (Abou Assi et al., 2017).

- **Sustainability and Cultivation:**

Research into sustainable cultivation practices can ensure that noni is harvested in an environmentally responsible manner, preserving its availability for future generations.

Morinda citrifolia, or noni, is a remarkable plant with a rich history and a plethora of potential health benefits. Its diverse range of bioactive compounds contributes to its antioxidant, anti-inflammatory, and immune-supporting properties. As traditional uses and modern applications continue to intertwine, *Morinda citrifolia* holds promise for further exploration in the realms of nutrition, herbal medicine, and pharmacology. With ongoing research and increasing interest, this tropical fruit may play a significant role in promoting health and well-being across diverse populations (Liang et al., 2024).

Rationale to consider *Morinda citrifolia* for PCOS

Morinda citrifolia is rich in lignan. Lignan is a polyphenol having low molecular weight. Various lignans are found to have a beneficial effect on obesity and obesity related conditions and diseases.

We can derive americanin A, isoprincepin, 3,3'-bisdemethypinoresinol, pisesamin 2,6-dicatechol etc lignan from the fruit part of Noni. (Su et al., 2005).

The testosterone decreasing potential also found in Lignan, they can bind the free testosterone in the 5α-reductase and enterohepatic circulations and then successfully helps in the conversion of dihydrotestosterone form testosterone. (Denis et al., 1999).

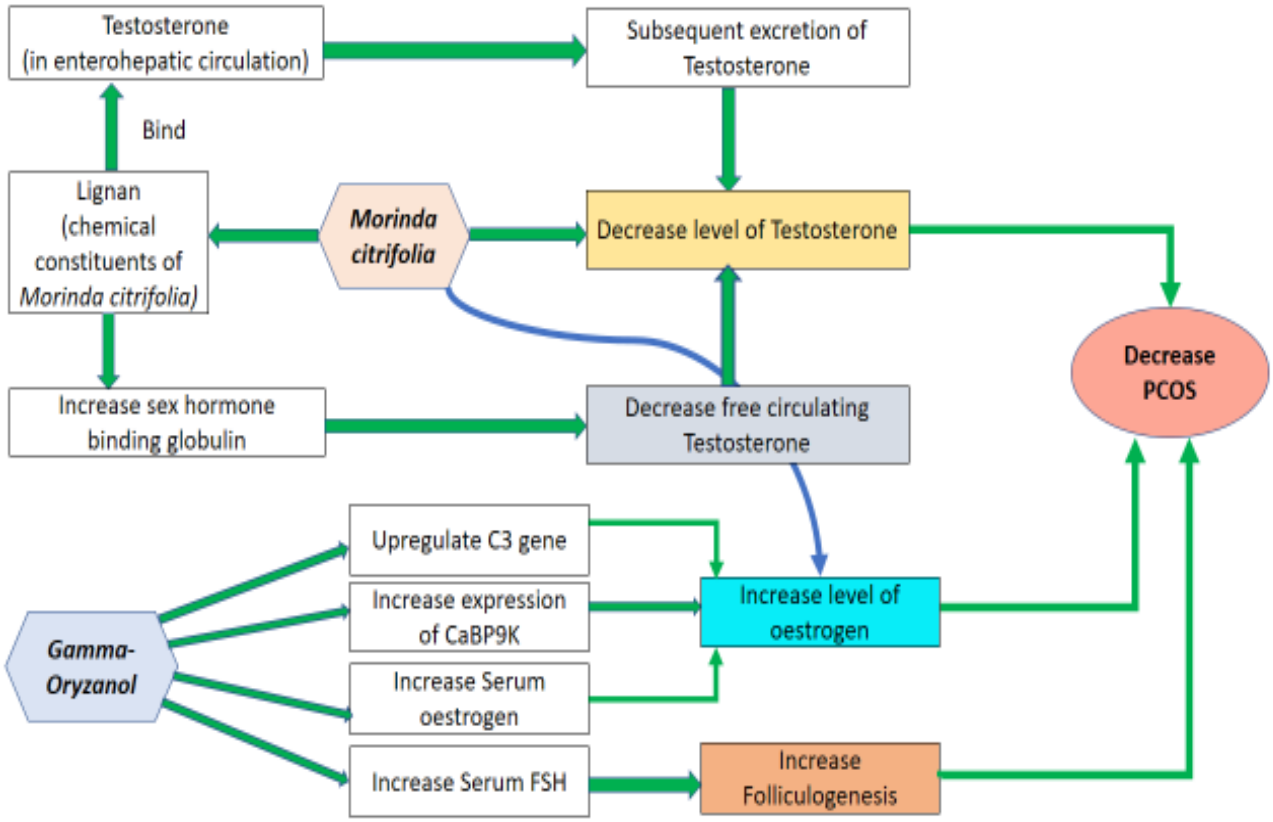
Lignan also act as a phytoestrogen or dietary estrogen for this reason, *Morinda citrifolia* found to Regulate oestrogen production and providing estrogenic effects in the human body (Ying Wang et al., 2004) (Chaisutatip et al., 2022).

The lignin present in *Morinda citrifolia* is able to bind testosterone in enterohepatic circulation which in turn promote a positive subsequent excretion of testosterone. This pathway helps to decrease the level of testosterone.

Lignan also increase the sex hormone binding globulin which decrease the free circulation testosterone. The level of testosterone also decreases by this way.

As a phytoestrogen noni can increase the level of estrogen. Phytoestrogen are the plant derived estrogen. In-vitro assay shows that noni has a higher binding affinity with ER-α and ER-β (Basar et al., 2006).

On a summery *Morinda citrifolia* target the main 2 clinical hallmark of PCOS by decreasing the level of testerone and increase the level of estrogen.



2.1. Rationale of the study

3.1. RATIONALE AND SCOPE OF THE STUDY

PCOS is a heterogeneous disorder with unclear pathophysiology. Presently available management strategies are not up to the mark as they are found to provide only symptomatic relief. So, it is necessary to implement more experimental strategies to combat this disorder.

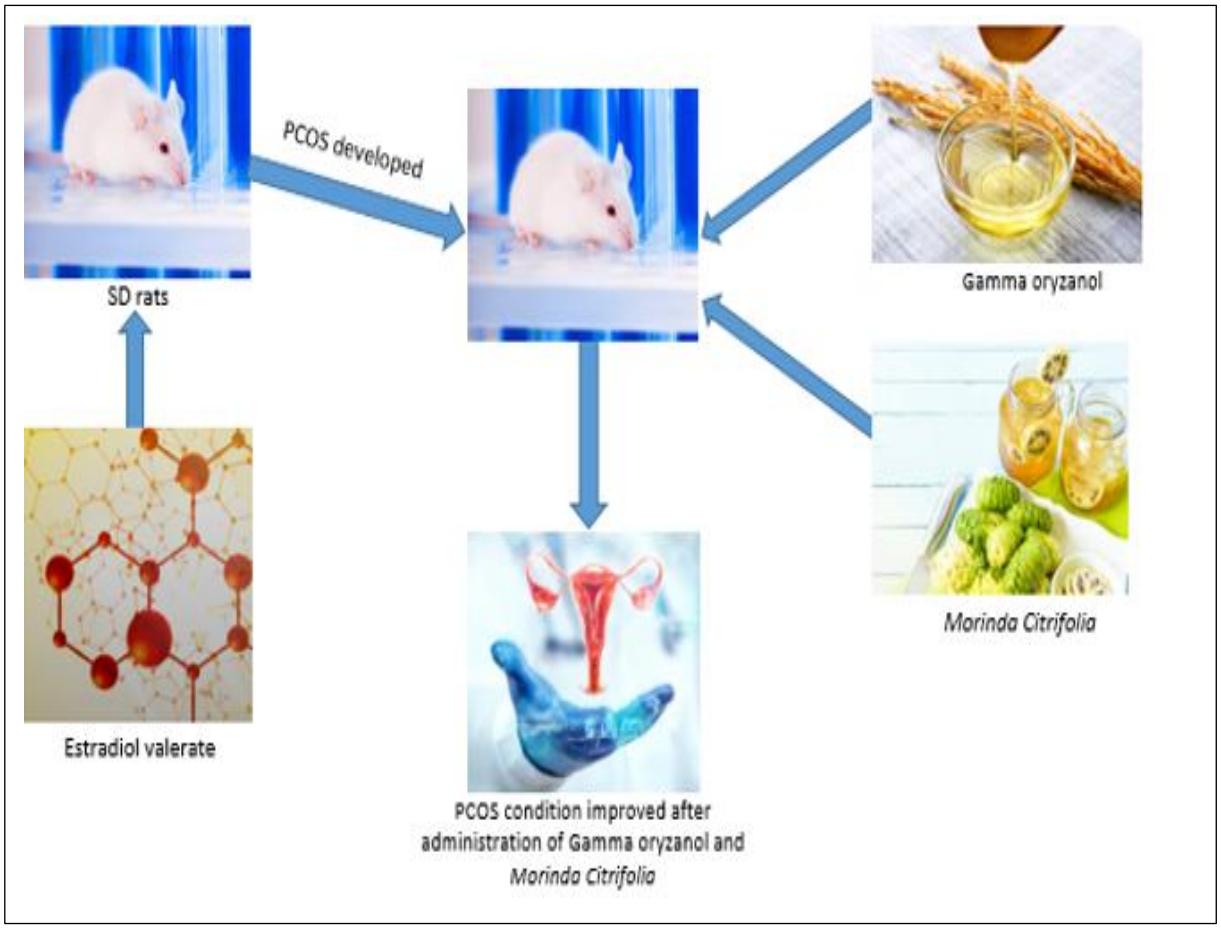
The dietary component, Gamma-oryzanol was able to establish its potential in the redaction of insulin sensitivity, redaction of oxidative stress, management of increase weight and can significantly increase oestrogen level and *Morinda citrifolia* was also able to establish its potential in the redaction of insulin sensitivity, regulation of testosterone level and promote the binding of sex hormone binding globulin. The effect of Gamma-oryzanol and *Morinda citrifolia* on these parameters indicates that there may be a causal relationship of Gamma-oryzanol and *Morinda citrifolia* with PCOS as, these parameters are the main clinical management strategies of PCOS. The role of Gamma-oryzanol and *Morinda citrifolia* in the management of PCOS still needs to be discovered. So as the dietary components, Rice bran oil and noni fruit could serve as potential candidates to manage PCOS.

3.2. AIM OF THE STUDY

Ameliorative effect of Gamma-oryzanol and *Morinda citrifolia* in Polycystic Ovary Syndrome using animal model.

3.3. OBJECTIVE OF THE STUDY

- To evaluate pharmacological effect of Gamma-oryzanol and *Morinda citrifolia* in polycystic ovary syndrome.
- To evaluate the combination effect of Gamma-oryzanol and *Morinda citrifolia* with the standard drug Clomifene Citrate in polycystic ovary syndrome.



3.1. Methodology of the study

3.4 PLAN OF WORK

Below steps are depicting how the plan of work executed:

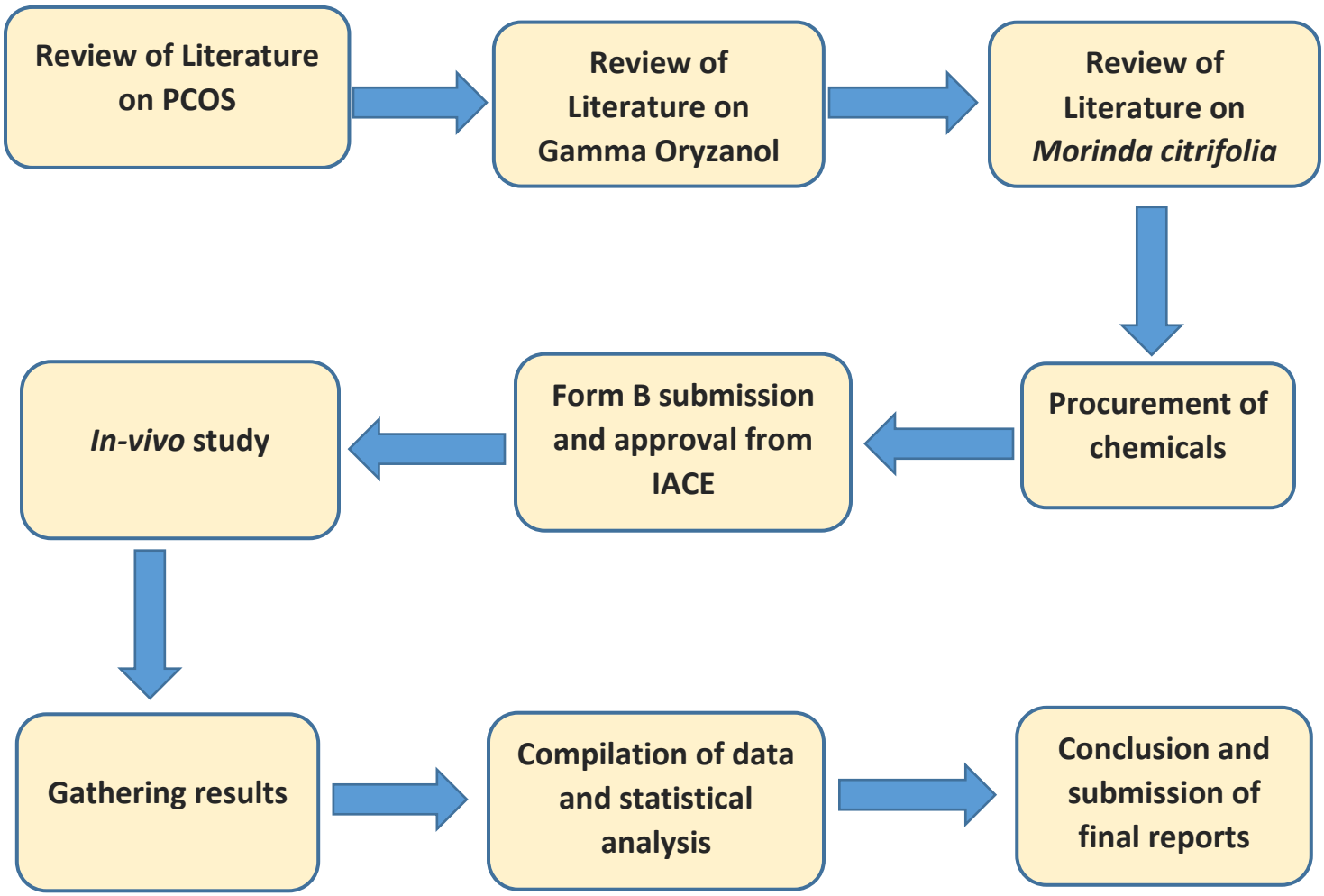


Fig. 3.4. Steps involved in the research work

4. EXPERIMENTAL WORK

4.1. EQUIPMENTS

Below **table 4.1.** Depicts the equipment used in the experiment

Table 4.1.: Equipment used for experimental work

EQUIPMENT	MANUFACTURER
Centrifuge	Remi Instruments, India
Deep freezer	Blue Star Ltd., India
Digital weighing balance	Contech Instruments Ltd., India
Refrigerator	Kelvinator International, India
Microscope	Remi Instruments, India
Rat testosterone, T ELISA Kit	EVERON life sciences
Rat Estrogen, E ELISA Kit	EVERON life sciences
Elisa reader	iMarkTM Microplate Reader, Bio-Red

4.2. CHEMICALS

The chemicals listed below were employed in the experimental procedures outlined in the study, playing a crucial role in the various stages of the research and analysis presented in **Table 4.2.**

Table 4.2.: Chemicals used for experimental work

Chemical	**Manufacturer**
Clomifene citrate (gm)	Shanghai Huirui Chemical Technology Co., Ltd.
Estradiol valerate (gm)	BB Chemicals
Gamma-oryzanol (gm)	Excolla Pharma Inc
***Morinda citrifolia* (gm)**	Xi'an Natural Field Bio-Technique Co., Ltd.

4.3. *IN VIVO* STUDY

4.4. ANIMALS

Sprague Dawley rats (87 Female + 10 Male), weighing 200-250 gm were used for the study. The rats were procured from the National Institute of Pharmaceutical Education and Research (NIPER), located in S.A.S. Nagar, Punjab, which operates as a CPCSEA-registered breeding facility ensuring ethical and regulated breeding practices. To reduce the potential stress associated with transportation, the animals were carefully transported by road in a dedicated institutional van, which provided a controlled environment for their journey. To provide comfort, free to movement, and to protect the animals from injury the animals were kept in the ideal size PP cages (Polypropylene). During the transportation sufficient food and water were arranged and it was ensured that animals got the access of food and water. The rats were kept at Lovely Professional University (Phagwara, Punjab) in the animal house. The normal humidity and temperature in a 12-hour light and 12-hour dark cycle was maintained throughout. The study was approved by Institutional Animal Ethics Committee (IAEC): IAEC/LPU/2020/78.

4.5. EXPERIMENTAL PROTOCOL

GROUP	TREATMENT	NO. OF ANIMALS
Group 1: Vehicle control	Olive oil 1 ml/kg	6
Group 2: Gamma-oryzanol *per se* treated group	Gamma-oryzanol 200 mg/kg p.o.	6
Group 3: *Morinda citrifolia per se* treated group	*Morinda citrifolia* 1000 mg/kg p.o.	6
Group 4: Disease control group	Estradiol Valerate 4 mg/kg	7
Group 5: Clomifene citrate treated group	Clomifene citrate 1 mg/kg in 0.5% CMC p.o.	6
Group 6: Gamma-oryzanol (100 mg/kg p.o. low dose) treated group	Gamma-oryzanol 100 mg/kg p.o.	7
Group 7: Gamma-oryzanol (200 mg/kg p.o. high dose) treated group	Gamma-oryzanol 200 mg/kg p.o.	7
Group 8: Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate treated group	• Gamma-oryzanol 100 mg/kg + • Clomifene citrate (1 mg/kg in 0.5% CMC p.o.)	7
Group 9: *Morinda citrifolia (500 mg/kg p.o. low dose)* treated group	*Morinda citrifolia* 500 mg/kg p.o.	7

Group 10: *Morinda citrifolia (1000 mg/kg p.o. high dose)* treated group	*Morinda citrifolia* 1000 mg/kg p.o.	7
Group 11: *Morinda citrifolia (500 mg/kg p.o. low dose)* + *Clomifene citrate* drug treated group)	• *Morinda citrifolia* 500 mg/kg + • Clomifene citrate (1 mg/kg in 00.5% CMC p.o.)	7
Group 12: Gamma-oryzanol + *Morinda citrifolia* drug treated group)	• Gamma-oryzanol 100 mg/kg + • *Morinda citrifolia* 500 mg/kg	7
Group 13: Gamma-oryzanol + *Morinda citrifolia* + Clomifene citrate drug treated group	• Gamma-oryzanol 100 mg/kg + • *Morinda citrifolia* 500 mg/kg + • Clomifene citrate (1 mg/kg in 0.5% CMC p.o.)	7
Group 14: Fertility study*	-	10

In Group 1, Olive oil 1 ml/kg was administered as a Vehicle control. Estradiol valerate was given to the group 4 at a single dose of 4 milligram per kg for the induction of PCOS like conditions (Amini, Tehranian, Movahedin, Ramezani Tehrani, et al., 2016). Clomifene citrate 1 mg per kg dissolve in 0.5% CMC was given in group 5. In group 6, Gamma-oryzanol 100 mg per kg was provided as low dose and in group 7 Gamma-oryzanol 200 mg per kg was provided as high dose.

In group 8, Gamma-oryzanol 100 mg per kg and Clomifene citrate was given in combination to observe the combination effect of standard with the test drug.

In group 9, Morinda citrifolia 500 mg per kg was provided as low dose and in group 10 Morinda citrifolia 1000 mg per kg was provided as high dose.

In group 11, Morinda citrifolia 500 mg per kg and Clomifene citrate was given in combination to observe the combination effect of standard with the test drug.

In group 12, Morinda citrifolia 500 mg per kg and Gamma-oryzanol 100 mg per kg was given in combination to observe the combination effect of both the test drugs.

In group 13, Morinda citrifolia 500 mg per kg, Gamma-oryzanol 100 mg per kg and Clomifene citrate was given in combination to observe the combination effect of all the treatment drugs.

In group 14, only male rats were taken for their utilization in fertility study.

All the administrations were given by oral. route. Evaluation of all the parameters required to establish PCOS condition (mentioned below) were performed on the treatment day of 14th.

The sample size taken between 6-7 animals per group to establish a statistical validity, 6-7 animals provide enough data to detect meaningful differences between groups. This sample size is ideal to controls variability which helps account for biological variation and reduces impact of outliers.

4.6. EVALUATIONS

4.7. Estrous cyclicity

Disruption in regular menstrual cyclicity is the major clinical outcome of PCOS. So, estrous cyclicity in rats was observed by the regular observation of the cell types present in vaginal smear.

Vaginal smears collected on each day provides throughout knowledge about the regularity of estrous cycle (OECD, 2009) . Rat estrous cycle is normally a 4 days cycle. Pro-oestrus (P) is the 1^{st} phase took place, followed by oestrus (O) which is the 2^{nd} phase, after that metoestrus (M) occure which is the 3^{rd} phase and lastly Di-oestrus (D) completes the cycle. These are the 4 phases of estrous cycle found in rats in the consecutive 4 days respectively.

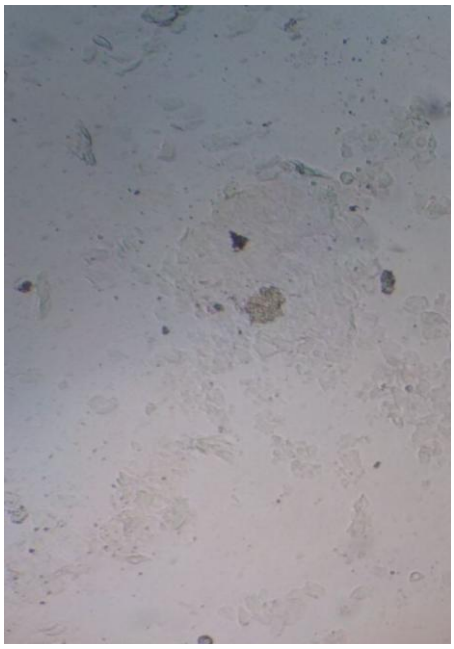
Pipette smear technique was applied for isolation of vaginal smear. As because in pipette smear technique the chance of pseudopregnancy is less in comparison with cotton swab technique along with that, pipette smear technique is easier to conduct as well as it gives a clarity in cell cytological examination on comparison to the cotton swab technique. A small amount of fluid was introduced into the vagina for flushing out cells of vaginal lining with the help of a pipette and dropped out one or two drops of vaginal cell suspension onto a glass slide.

The rat was held by rounding the thorax, by facing the ventral surface uphill for the easy reach of vaginal orifice. 0.9% w/v NaCl basically using the Normal Saline and taken 0.2% in pipette tip and administered inside vaginal cannel of the rats, waited for 2-3 seconds and then the saline is taken out with the help of same pipette tip. This process is followed to take vagina smear daily to determined estrous cycle phases and also to confirm the copulation. After collecting the smear, it was placed into glass slide and observed under microscope. A cover slip is also added to lock the sample in the place.

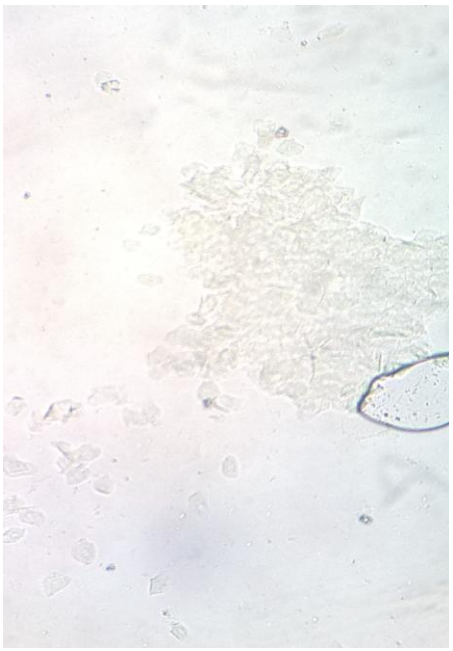
The cell type of vaginal smear was changed in each and every phase of estrous cycle which is depicted in Fig. 4.3. The change in vaginal cytology along with the ovarian and uterine events in each and every phase of this cycle is listed in Table 4.3 and fig 4.1 depicts the cytological changes in the each and every phase of the estrous cycle.

Table 4.4.: The Change in Vaginal Cytology and Ovarian and Uterine Events in Accordance With the 4 Phases of Estrous Cycle

PHASE	VAGINAL CYTOLOGY	OVARIAN & UTERINE EVENTS
P= Pro-estrous	Epithelial cells: The rounded cells mainly, some of them are showing early signs of cornification (nucleated).	• Follicles start to grow. • Endometrium starts to develop. • Corpus Leuteum gets degenerated.
O= Estrous	Cornified cells: Large cells, arranged in clumps.	• Follicles became mature. • Oestrogen at high peak. • Animal becomes sexually receptive.
M= Met-estrous	Leucocytes: Large no. of leucocytes cells. A small amount epithelial cells also present which are generally non nucleated.	• Formation of Corpus Leuteum. • Corpus Leuteum starts secreting progesterone. • Uterine linings begin to appear.
D= Di-estrous	Mainly small rounded leucocytes but with small no of epithelial and cornified cells.	• Degradation of Corpus Leuteum. • Restoring of endometrium. • Uterine lining gets resorbed.

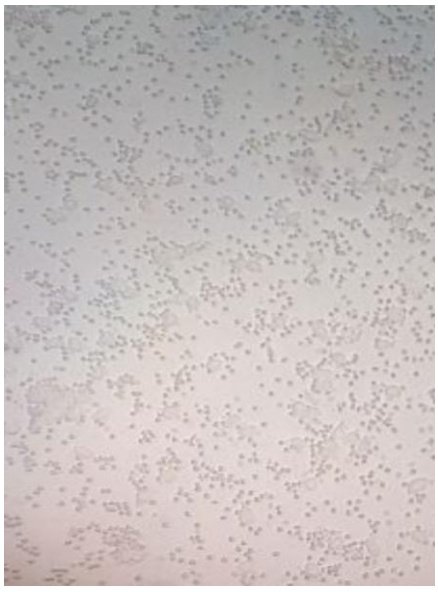


PRESTROUS



ESTROUS

DIESTROUS



METESTROUS

Fig 4.1: Different phases of estrous cycle

4.8. Fertility study

a. Each day during the study phase, the vaginal smear was evaluated for all the female rats. The rats show proestrus phase by the vaginal cytology (because ovulation take place almost in the midnight immediately after the ending of the pro-estrous phase on which female rats become sexually active for the male one) were identified and then after 5:00pm afternoon, the rats in proestrus were introduced with male fertile male rats for over-night to induce copulation. On the following morning, again the vaginal smear was taken to confirm the presence of sperms inside vaginal lavage (fig 4.2). After sacrificing animals, fertility rate was assessed for each animal. The implantation site count measures how many embryos have successfully attached to the uterus, offering a direct indication of reproductive success. It reflects not only the viability of embryos and effective fertilization but also the readiness of the uterine environment to support pregnancy. This count is influenced by hormonal balance and overall reproductive health. Changes in implantation site numbers help evaluate how various treatments or conditions affect fertility and early pregnancy outcomes.

b. After scarification, the uterus was removed from body and the number of implantations sites (Fig 4.3) were observed for each female rats (Thakare et al., 2009).

c. Body weight and ovarian weight changes were also evaluated (Amini, Tehranian, Movahedin, Ramezani Tehrani, et al., 2016).

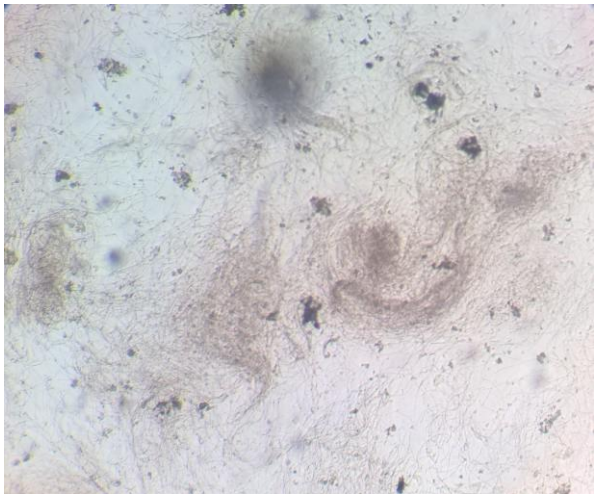


Fig 4.2: Presence of Sperm inside vaginal smear confirms the copulation

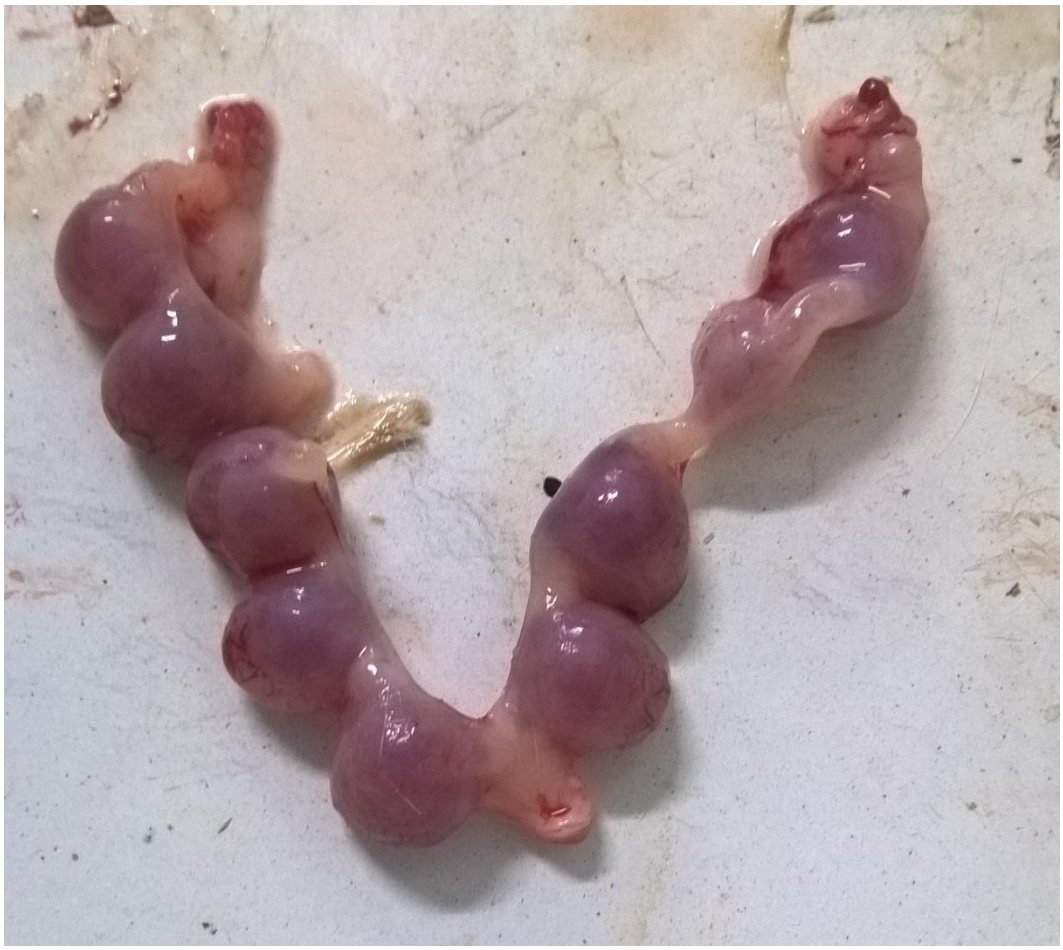


Fig 4.3: Presence of Implantation sites on both uterus

4.9. Post-mortem evaluations

Euthanasia: The animals were sacrificed using cervical dislocation method. The animals were placed in recumbence by dorsal side. A needle of 23-27 gauge was attached to the syringe. Palpitation of heart need to be identified which can be found in the thoracic wall of left side. The needle was inserted at an angle of 30-40^{0}C through the diaphragm and inserted to the heart directly. Now the plunger was retracted until the blood started to ooze out in the syringe. The required amount of blood was collected with the help of the above explained cardiac puncture method (University Veterinarian & Animal Resources, 2017).

4.9.1. Histopathological analysis- Both of the ovaries were undergoing a histopathological evaluation. Ovaries (both ovaries from each rat) were immobilized minimum for a day

using 10% formaldehyde. Corresponding to the standard protocols, the tissue was processed and after that, 5 μm sections taken, blocked and fixed into paraffin in the longitudinal direction. Tissues of ovaries sliced after completion of fixing and blocking longitudinally in paraffin. Haematoxylin and eosin both used for the staining of the tissue. Presence of cystic type of formations were observed in both ovaries in ×100 and ×40 magnifications (Amini, Tehranian, Movahedin, Tehrani, et al., 2016).

4.9.2. Biochemical studies

- **Hormone levels:** The blood samples from euthanized female rats form each group was collected on the terminal day of the treatment. The collected blood samples were allowed to clot for about 1 hour at room temperature followed by centrifugation for 10 min at 14000 *g* to obtain the serum. The serum was collected and was preserved at −20°C for further analysis. Further the serum estradiol and testosterone levels of estradiol valerate-treated female rats was measured using ELISA kit according to the manufacture's protocol (Pillai et al., 2010).

The ELISA (Enzyme-Linked Immunosorbent Assay) principle is a widely used laboratory technique for detecting and quantifying proteins, antibodies, or hormones in a sample.

Below is the basic ELISA principle.

Coating: A solid surface (often a microplate) is coated with an antigen or antibody that specifically binds to the target molecule.

Blocking: After the coating process, any remaining unoccupied sites on the surface are blocked using a protein solution to effectively prevent nonspecific binding and ensure that only specific interactions occur during the experiment.

Sample Addition: The sample, containing the target molecule, is subsequently introduced into the system. If the target molecule is present, it will selectively bind to the antibodies or antigens that have been coated on the surface. This binding forms a stable antigen-antibody complex, which can then be detected and analyzed to confirm the presence of the target.

Detection: A secondary antibody, conjugated with an enzyme, is then added to the system. This secondary antibody specifically binds to the target molecule, forming a sandwich complex in the

case of a sandwich ELISA. The binding of the secondary antibody to the target molecule enhances the specificity of the detection, allowing for the subsequent measurement of the enzyme activity linked to the complex.

Substrate Addition: A substrate for the enzyme is then introduced into the system. The enzyme catalyzes a reaction that leads to the production of a detectable signal, typically in the form of a color change. This color change serves as an indicator of the presence and amount of the target molecule, providing a measurable output for analysis.

Measurement: The intensity of the generated signal, such as the color change, is then measured, typically using a spectrophotometer. The absorbance or optical density (OD) readings obtained correlate directly with the concentration of the target molecule present in the sample, allowing for quantitative analysis.

In this study we evaluated hormonal analysis with the help of ELISA. Estrogen and testosterone level were evaluated as these are two major hormones associated with the PCOS

.

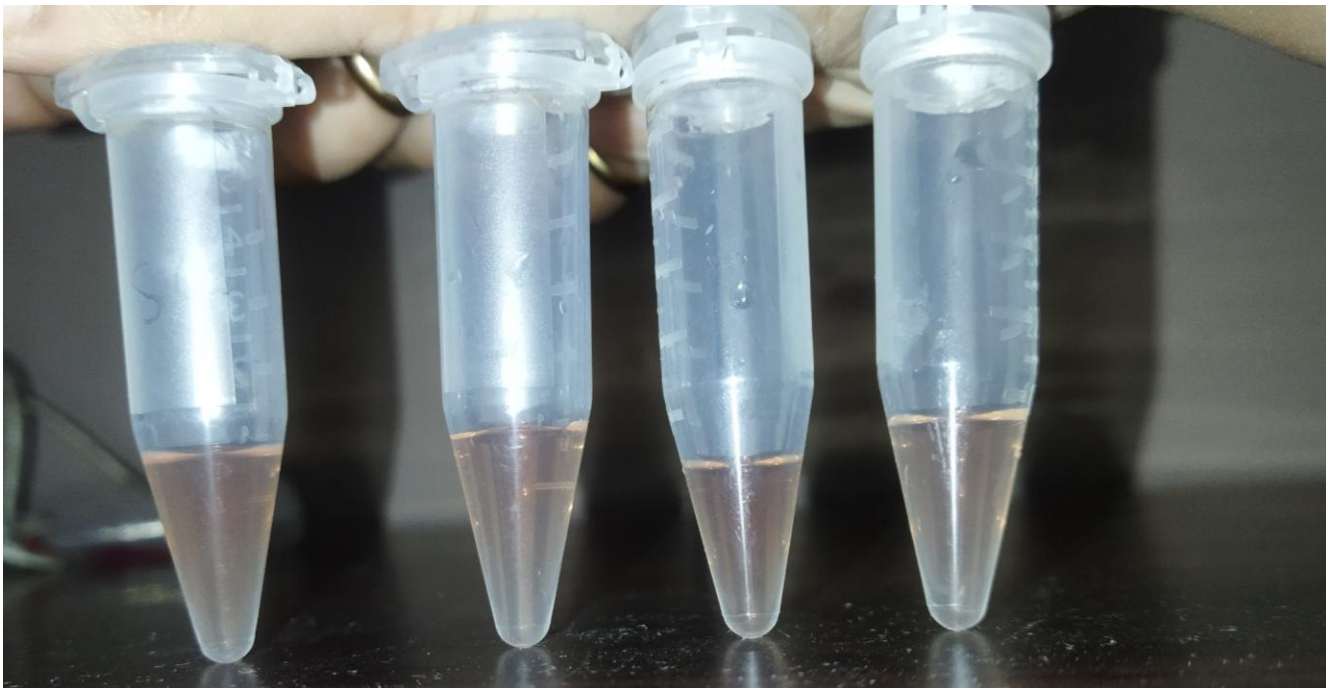


Fig 4.4. Blood serum of female rats

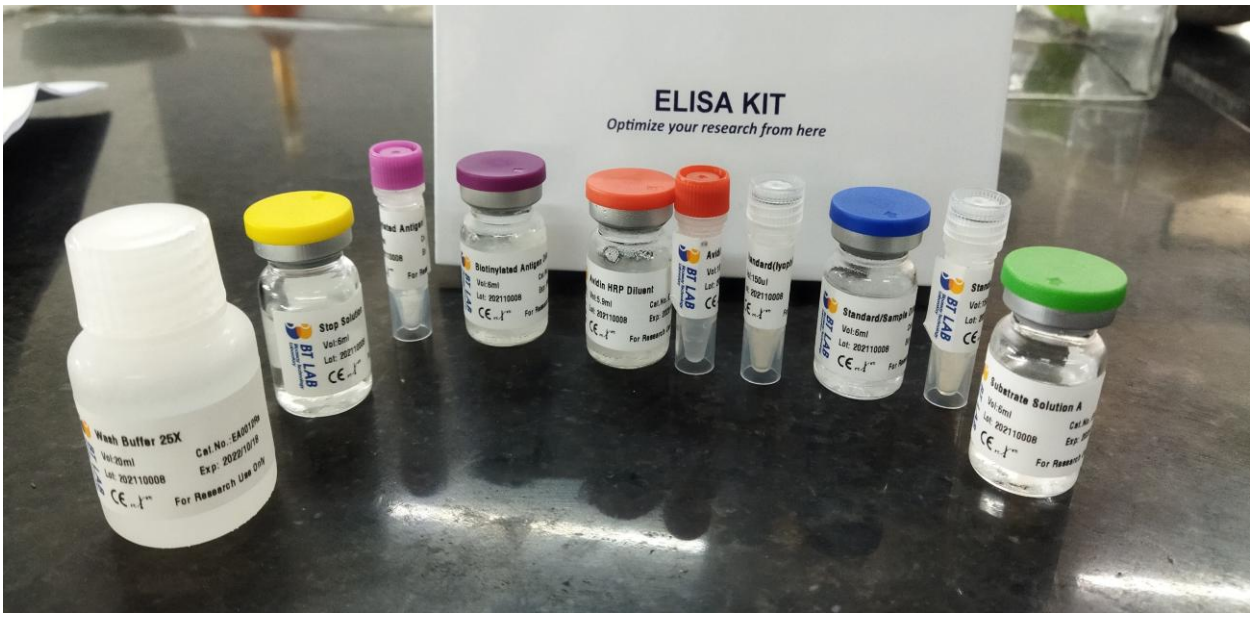


Fig 4.5. Estrogen ELISA KIT

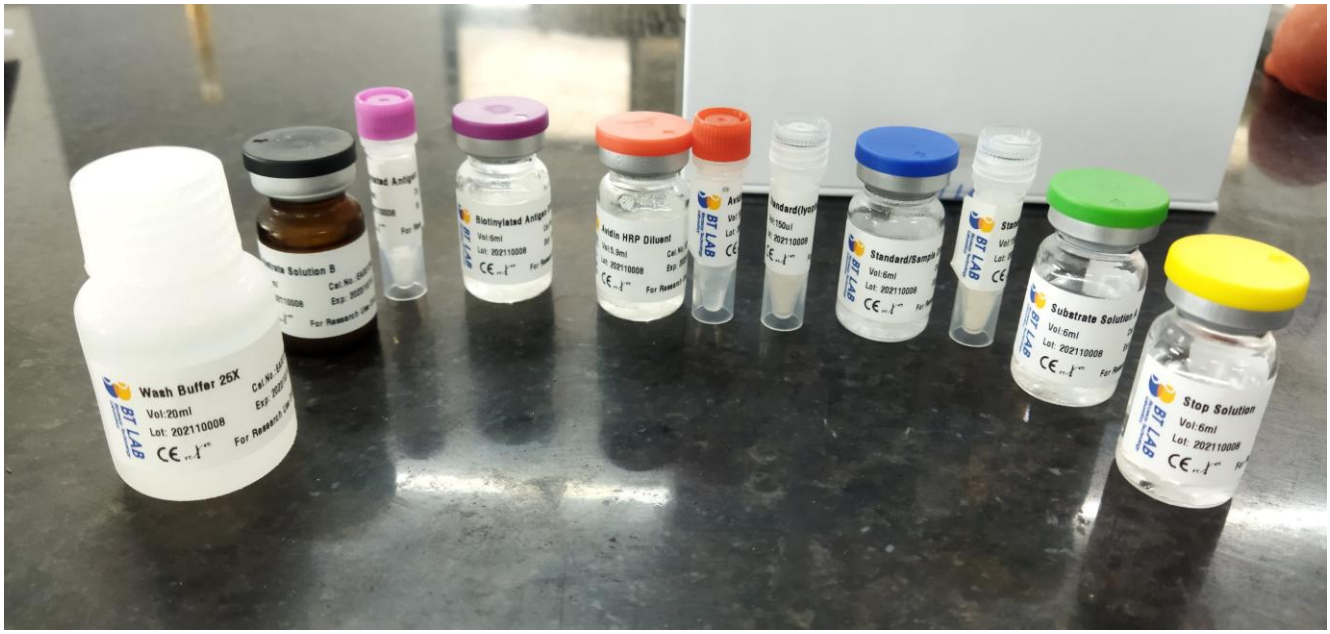


Fig 4.6. Testosterone ELISA KIT

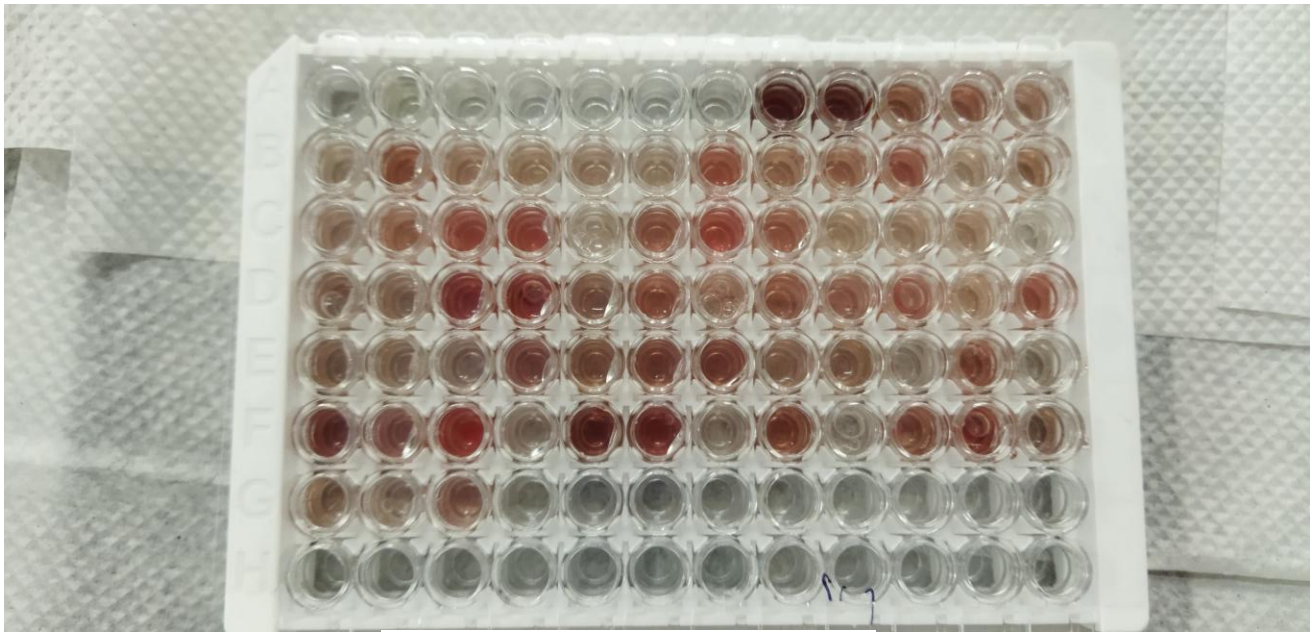


Fig 4.7. Assay procedure

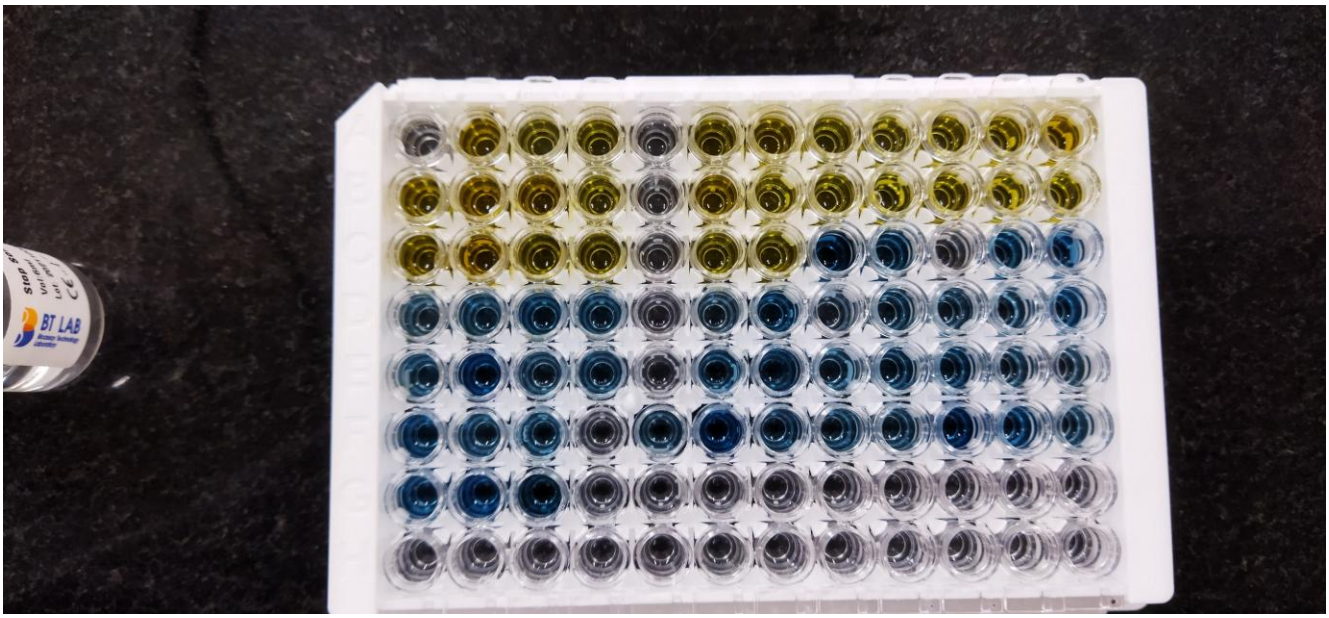


Fig 4.8. Assay procedure

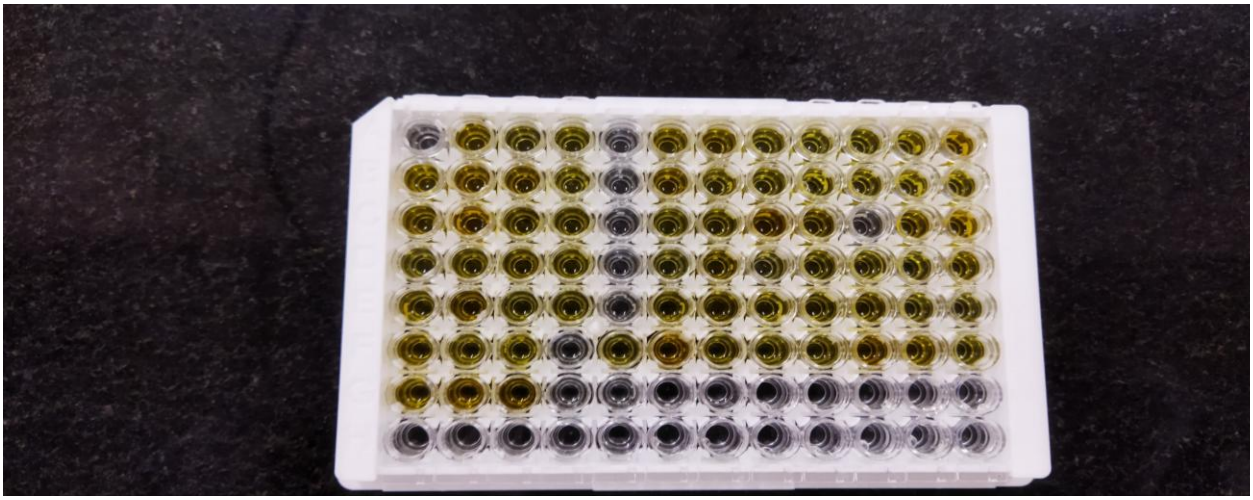


Fig 4.9. Assay procedure

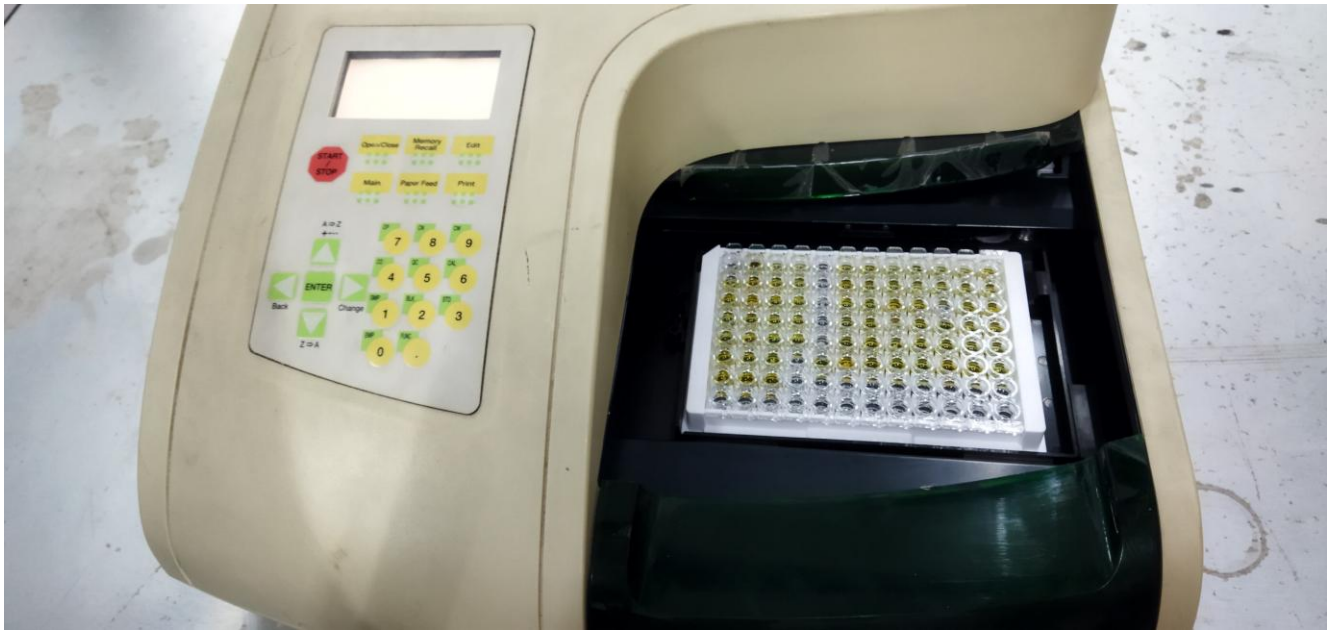


Fig 4.10. ELISA Reader: Determining optical density

- **Anti-oxidant assay**

The levels of SOD, GSH, and CAT was measured in ovarian homogenate to determine the anti-oxidant capacity of ovaries. Ovarian homogenate (10% w/v) was prepared using Tris-HCl buffer (0.1 M) of pH 7.8 and was centrifuged for 60 min at 8,000 *g*. Afterwards, the supernatant was collected to determine below enzymatic parameters (Hong et al., 2019)

SOD levels

Superoxide dismutase, SOD is an antioxidant enzyme which catalysed the partitioning of 0_2^- (superoxide radicle) into 0_2 and H_20_2. 0_2^- is a destructive radicle for a cell which causes oxidative stress. The appropriate levels of SOD in the body are a prime concern as the deviation leads to increase the oxidative stress, which in turn promotes and amplifies disease conditions. The reactions are as below.

$$2H^+ + 20_2^- \rightarrow 0_2 + H_20_2$$

SOD catalysed 0_2^- for Cu, ZnSOD through one of the below reactions:

- Cu^{2+}-SOD + 0_2^- → Cu^+-SOD + 0_2 (copper redacted, superoxide oxidised)
- Cu^+-SOD + 0_2^- + $2H^+$ → Cu^{2+}-SOD + H_20_2 (superoxide redacted, copper oxidised)

SOD activity was evaluated by following the below procedures (Misra & Fridovich, 1972) (Sun et al., 1988)

For the assay of SOD activity, the below solutions were used:

- Standard SOD solution: 4 mg of Cu, ZnSOD was dissolved in 50 ml of double distilled water, refrigerated until use and diluted to 600µl with double distilled water before use.
- Standard MnSOD solution: 1.1gm of MnSOD was dissolved in 1 litre of double distilled water to make 1.1g/l.
- Xanthine oxide solution: 20µl of Xanthine oxide was dissolve in 2.0 ml of ice-cold ammonium sulphate (2mol/l). Final concentration retained at 167 U/l.
- Reagents utilized in SOD assay: Below table consist of 5 chemicals were mixed to prepared SOD assay reagents.

Table 4.5.: Reagents used in SOD activity

Sl no	Reagent	Concentration	Volume required
1	Xanthine solution	0.3 mmol/l	40 ml
2	EDTA solution	0.6 mmol/l	20 ml
3	NBT (Nitroblue tetrazolium) solution	150 µmol/l	20 ml
4	Na_2C0_3	400 mmol/l	12 ml
5	Bovine serum albumin	1g/l	6 ml

SOD assay:

- 2.45 ml SOD reagent was added in each test tube.
- 0.5 ml Cu, ZnSOD and MnSOD was added.
- The test tubes were placed in a water bath at 25°C.
- 50 ml of Xanthine oxidase solution were added and incubated for 20 mints.
- 1 ml of 0.8 mmol/l $CuCl_2$ solution were added to terminate the reaction.
- At 560 nm, the absorbance was observed. % of inhibition was calculated by the below formula.

$$\% \text{ of inhibition} = \frac{-(A\ blank - A\ sample)}{A\ blank} * 100$$

- The enzyme concentration that inhibited 50% of oxidation was designated as one enzymatic unit (1 UI) at 25°C.

GSH activity

As per the below procedure the GSH activity were measured using spectrophotometer. (Habig et al., 1974) (Rahman et al., 2007)

Glutathione S-transferase plays a key role in the protection of cells by neutralization of reactive oxygen species by the below reactions:

$$2GSH + R_2O_2 \rightarrow GSSH + 2ROH$$

$$GSH + R\cdot \rightarrow \frac{1}{2} GSSG + RH$$

Glutathione present in 2 states, reduced known as GSH and Oxidised known as GSSG. The ratio of GSSH and GSH depicts oxidative stress. This ratio is proportional to the oxidative stress. Glutathione is catalysed by the below reactions:

$$NADPH + GSSH + H_2O \rightarrow 2GSH + NADP^+ + OH^-$$

From the protectable proteins RSH and GSH, disulfide is produced by the below reaction:

$$RSH + GSH + [O] \rightarrow GSSR + H_2O$$

For the assay of GSH activity the below solutions were used:

- KPE Buffer: 0.1 M potassium buffer was added with 5nM of EDTA solution. 2 solutions were made.
  Solution A: 6.8 g of KH_2PO_4 was dissolved in 50 ml of dH_20, kept it in 4°C.
  Solution B: 8.5 g of KH_2PO_4 was dissolved in 500 ml of dH_20, kept it in 4°C.

16 ml of Solution A was added to 84 ml of Solution B. 0.327 g EDTA was added to that mixture and pH was adjusted to 7.5.

- DTNB: 2 mg of DNTB dissolved in 3 ml of KPE.
- NADPH: 2 mg of βNADHP dissolved in 3 ml of KPE.
- GR: 40 µl of glutathione reductase (GR 250units ml^{-1}) in 3 ml of KPE.
- GSH standard: 1 mg of GSH^{-1} was dissolved in KPE.
- GSSG Standard: 2.01 mg of $GSSG^{-1}$ was dissolved in KPE.

GSH assay:

- 700 µl of KPE buffer was added to a 1 ml cuvette.
- Replaced it with 100 µl of sample.

- Equivalent volume of DTNB, GR was added to 120 µl of this mixture.
- After 30s, 60 µl of βNADHP was added to it.
- At 412 nm the absorbance was observed.
- Amount of GSH was derived from a standard curve.
- The formula for GSH concentration:

  GSH concentration → Total GSH-GSSG

  However, data was expressed as GSH equivalent (GSH+2GSSG).
- The unit for GSH concentration is considered as nM or µM per mg of protein gm^{-1} of tissue or $nM/10^6$ cells.

Catalase (CAT) activity

Catalase is an enzyme which is also act as an antioxidant. Catalase helps in the decomposition of H_20_2. H_20_2 is a bi-product produced by various cellular mechanisms. It is necessary to decompose H_20_2 into other components as H_2O_2 causes oxidative stress.

Catalase is an enzyme which catalyse H_2O_2 into water and oxygen by the below reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Besides that, catalase also has a peroxidic activity where it oxidises H doners like ethanol, phenol, methanol etc. The reaction of catalase having peroxidic activity is as below:

$$ROOH + AH_2 \rightarrow H_2O + ROH + A$$

The decomposition of H_2O_2 initially follows first order reaction with H_2O_2 concentration between 0.01 M and 0.05 M. The rate constant (k) can be used as a direct measurement of catalase concentration.

$$k = (1/\Delta t)\ In\ (S_1 - S_2) = (2.3//\Delta t)\ (log\ S_1 - S_2)$$

where, $\Delta t = t_1 - t_2$ (time interval) S_1 and $S_2 = H_2O_2$ concentration at times t_1 and t_2.

In this study the specific catalase activity (k_1') is obtained by dividing rate constant (k) by molar concentration of catalase (e).

$$k_1' = k/e \text{ Liters mol}^{-1} \text{ sec}^{-1}$$

Activity of CAT were evaluated using spectrophotometer as described by (Aebi, 1984).

For the assay of CAT activity, the below reagents were used:

Solution A: 6.81 g of KH_2PO_4 was dissolved in 50 nM of phosphate buffer.

Solution B: 8.90 g of Na_2HPO4, $2H_2O$ was dissolved in 50 nM of phosphate buffer.

Solution A and B were mixed at a ratio 1:15 (v/v). Distilled water was added to make up to 1000 ml.

Hydrogen peroxide 30 nM: 0.34 ml 30% H_2O_2 was diluted with phosphate buffer to 100 ml.

CAT assay:

- In, 50 mM potassium phosphate buffer (pH of 7.0) aliquot was added.
- In 2 ml of enzyme solution, 1 ml of H_2O_2 was added. The mixture was kept at 20°C. H_2O_2 was added to initiate enzymatic reaction.
- At 240 nm, by the disappearance of H_2O_2 the absorbance is observed.
- The enzyme quantity necessary to induce the disappearance of H2O2 was referred to as Units (U) per milligram of protein, with 1 Unit corresponding to the decomposition of 1 mmol of H_2O_2 per minute.
- The difference in absorbance/unit time is a measure of the catalase activity.

4.10. Statistical Analysis

The findings were presented as the mean ± standard deviation (SD) to provide a clear representation of the data distribution. To determine the statistical significance of the results, a one-way analysis of variance (ANOVA) was initially performed. Following this, post-hoc comparisons were conducted using the Tukey test to identify specific differences between the groups (Sigma Plot, 14.0). Statistically significant for all comparisons parameters kept as P-values <0.05, <0.01 and <0.001.

5.1. Estrous cyclicity`

In the vehicle control, gamma oryzanol per se, *Morinda citrifolia* per se it was observed that, a perfect "W" shaped graph due to absence of disruption, each day represent each phase same as a healthy rat.

For disease control group the disruption is significant in comparison with vehicle control, gamma oryzanol per se, *Morinda citrifolia* per se group. Clomifene citrate group depicts a statistically significant improvement.

There was a significant improvement with Gamma oryzanol treatment (Both doses) in the comparison with the disease control group. The improvement found to be non-significant for Gamma oryzanol (both doses) in comparison with Clomifene citrate but Gamma oryzanol with Clomifene citrate group portrays a significant (statistically) improvement as compared disease control, standard drug (Clomifene citrate) and Gamma oryzanol treatment (Both doses) groups.

There was a significant improvement with *Morinda citrifolia* treatment (Both doses) in the comparison with the disease control group. The improvement found to be significant for *Morinda citrifolia* (both doses) in comparison with Clomifene citrate also, *Morinda citrifolia*+ Clomifene citrate group depicts a statistically significant improvement as compared disease control and *Morinda citrifolia* treatment (Both doses) groups.

Gamma oryzanol+ *Morinda citrifolia* group depicts a statistically significant improvement in comparison with disease control, standard drug, Gamma oryzanol (Both dose) groups, *Morinda citrifolia* (Both dose) groups.

Gamma oryzanol+ *Morinda citrifolia*+ Clomifene citrate group depicts a statistically significant improvement in comparison with disease control, standard drug, and test drug combination group (GO+MC) (Fig 8)

The improvement of estrous cyclicity for the group receiving the combined treatment of gamma oryzanol and clomifene citrate shows 0.08-fold increase as compared to standard drug, similarly *Morinda citrifolia*+ Clomifene citrate group shows 1.025-fold increase while compared to standard drug.

The combination of Gamma oryzanol + *Morinda citrifolia* group shows 0.5-fold improvement of estrous cyclicity as compared to standard drug, similarly Gamma oryzanol+ *Morinda citrifolia*+ Clomifene citrate group shows 0.1-fold improvement of estrous cyclicity while compared to standard drug.

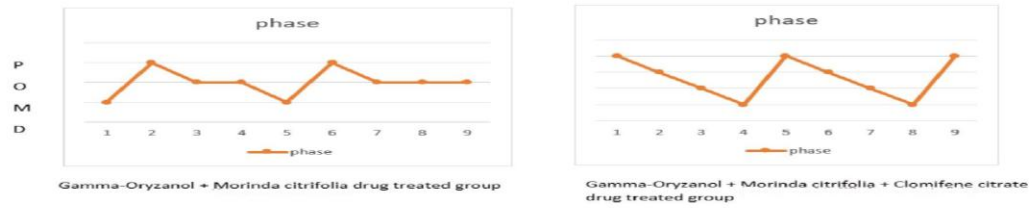


Fig 5.1.: Estrous cyclicity of the groups

Table 5.1.: Estrous cyclicity of the groups

Group	**Disruption score of estrous cyclicity**
Vehicle control	0
Gamma-oryzanol per se	0
Morinda citrifolia per se	0
Disease control	1.8^{***}
Clomifene citrate	$1^{\#\#\#}$
Gamma-oryzanol (100 mg/kg p.o. low dose)	$1.4^{\#}$
Gamma-oryzanol (200 mg/kg p.o. high dose)	$1.2^{\#\#\#}$
Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate	$0.08^{\#\#\#\wedge\wedge\wedge\%\%\%}$
Morinda citrifolia (500 mg/kg p.o. low dose)	$1.6^{\#\wedge\wedge\wedge}$
Morinda citrifolia (1000 mg/kg p.o. high dose)	$1.5^{\#\#\wedge}$
Morinda citrifolia (500 mg/kg p.o. low dose) + Clomifene citrate	$1.03^{\#\#\#\$\$\$}$
Gamma-oryzanol + *Morinda citrifolia*	$0.5^{\#\#\#\wedge\wedge\wedge\%\%\%\$\$\$}$
Gamma-oryzanol + *Morinda citrifolia* (500 mg/kg p.o. low dose) + Clomifene citrate	$0.1^{\#\#\#\wedge\wedge@}$

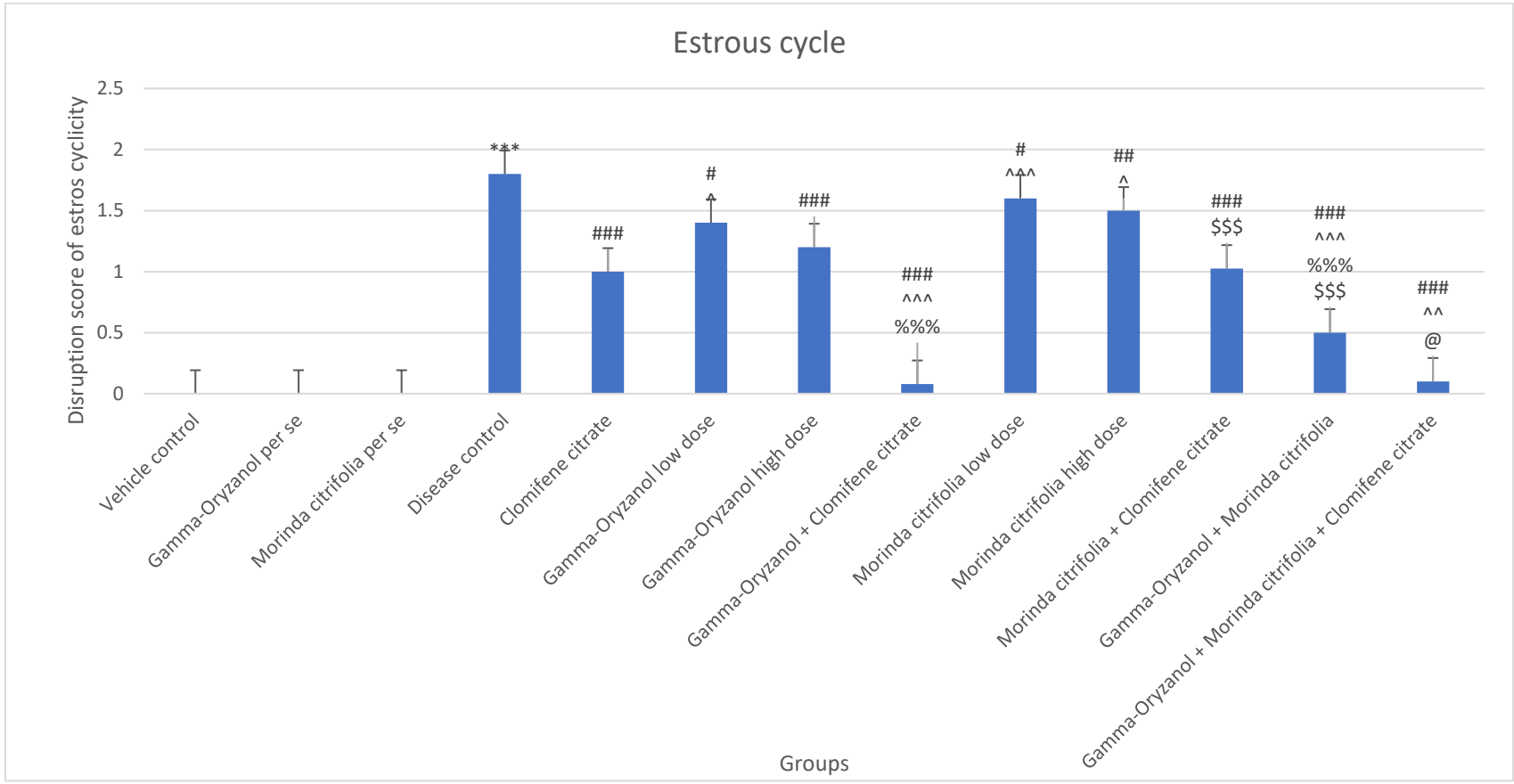


Fig 5.2.: Disruption of estrous cycle in groups: The data are expressed as the mean values ± standard error of the mean (SEM) to give an indication of the variability and precision of the measurements. Statistical significance was evaluated based on the p-value thresholds: *p < 0.05 was considered statistically significant, **p < 0.01 indicated a highly significant difference, and *p < 0.001 denoted an extremely significant difference between the compared groups**."*** Comparison between control to *per se* and disease control group, # Comparison between disease control group to treatment groups, ^ Comparison between standard drug to treatment groups, **%** Comparison between gamma oryzanol treated groups to combination treatment groups (GO+CC and GO+MC), **$** Comparison between *Morinda citrifolia* treated groups to combination treatment groups (MC+CC and GO+MC), **@** Comparison between combination treatment groups (GO+MC *vs*. GO+MC+CC)

5.2. Biochemical studies- Estradiol and testosterone concentration (in blood serum) were examined by using ELISA lit. Antigen, Horseradish peroxidase was used for labelling the enzyme to compare unlabelled antigen on the ELISA plates. At 450 nm, the absorbance was evaluated for samples and standard. (Pillai et al., 2010).

5.3. Estrogen: Statistically significant decrease the concentrations of estrogen were assessed and observed in all of the different treatment groups., in contrast with the group that underwent treatment with estradiol valerate and for all the combination groups. Gamma oryzanol and Noni both exhibit significant decrease in estrogen level while compared with disease control group. There was a statistically significant decrease in the level of estrogen in the group administered a treatment regimen consisting of both gamma oryzanol and clomifene citrate in comparison with the group that received the standard treatment regimen of clomifene citrate. There was a statistically significant decrease in the level of estrogen in Noni and clomifene citrate combination treatment group in comparison with the group that received the standard treatment regimen of clomifene citrate. Estrogen level was decreased in Gamma-oryzanol+ Noni+ Clomifene citrate group while compared with Gamma-oryzanol+ Noni but the decrease did not demonstrate statistical significance.

The increase of estrogen level for the group receiving the combined treatment of gamma oryzanol and clomifene citrate shows 0.6-fold increase as compared to standard drug, similarly *Morinda citrifolia*+ Clomifene citrate group shows 0.72-fold increase while compared to standard drug.

The combination of Gamma oryzanol + *Morinda citrifolia* group shows 0.56-fold increase in of estrogen level as compared to standard drug, similarly Gamma oryzanol+ *Morinda citrifolia*+ Clomifene citrate group shows 0.5-fold increase while compared to standard drug.

Table 5.2.: Serum concentrations of Estrogen in groups

GROUP	CONCENTRATION (ng/ml)
Vehicle control	9020.81±0
Gamma-oryzanol per se	9760.39±3984.66
Morinda citrifolia per se	9760.39±5635.16
Disease control	25760.39±14872.77***
Clomifene citrate	20009.56±11552.53##
Gamma-oryzanol (100 mg/kg p.o. low dose)	19876.21±11475.54##
Gamma-oryzanol (200 mg/kg p.o. high dose)	18876.21±11475.54##
Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate	12054.35±6959.58###^%
Morinda citrifolia (500 mg/kg p.o. low dose)	22484.56±12981.47#
Morinda citrifolia (1000 mg/kg p.o. high dose)	19884.56±11480.36##
Morinda citrifolia (500 mg/kg p.o. low dose) + Clomifene citrate	14306.22±8259.70###^^^$$$
Gamma-oryzanol + *Morinda citrifolia*	11304.08±6526.418###^^^%%%$$$
Gamma-oryzanol + *Morinda citrifolia* (500 mg/kg p.o. low dose) + Clomifene citrate	9260.39±5208.16###^^^

5.4. Testosterone: Statistically significant decrease in the concentrations of testosterone were assessed and observed in all of the different treatment groups., in contrast with the group that underwent treatment with estradiol valerate and for all the combination groups. Gamma oryzanol and Noni both exhibit significant decrease in testosterone level while compared with disease control group. Testosterone level was decreased in Gamma-oryzanol + Noni + Clomifene citrate group while compared with Gamma-oryzanol + Noni but the decrease did not demonstrate statistical significance.

The level of testosterone increases in the group receiving the combined treatment of gamma oryzanol and clomifene citrate shows 0.82-fold increase as compared to standard drug, similarly *Morinda citrifolia*+ Clomifene citrate group shows 0.90-fold increase while compared to standard drug.

The combination of Gamma oryzanol + *Morinda citrifolia* group shows 0.7-fold increase in testosterone level as compared to standard drug, similarly Gamma oryzanol+ *Morinda citrifolia*+ Clomifene citrate group shows 0.5-fold increase while compared to standard drug.

Table 5.3.: Serum concentrations of Testosterone in groups

GROUP	CONCENTRATION (ng/ml)
Vehicle control	221.05±127.62
Gamma-oryzanol per se	213.05±123
Morinda citrifolia per se	221.05±127.62
Disease control	772.86±446.21***
Clomifene citrate	480.81±277.59###
Gamma-oryzanol (100 mg/kg p.o. low dose)	620.06±357.99##
Gamma-oryzanol (200 mg/kg p.o. high dose)	544.48±314.36###
Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate	398.35±229.99###%%%
Morinda citrifolia (500 mg/kg p.o. low dose)	640.16±369.59#
Morinda citrifolia (1000 mg/kg p.o. high dose)	570.64±329.46##
Morinda citrifolia (500 mg/kg p.o. low dose) + Clomifene citrate	436.97±252.28###$$$
Gamma-oryzanol + *Morinda citrifolia*	336.46±194.25###%%%
Gamma-oryzanol + *Morinda citrifolia* (500 mg/kg p.o. low dose) + Clomifene citrate	230.92±133.32###^^^$$

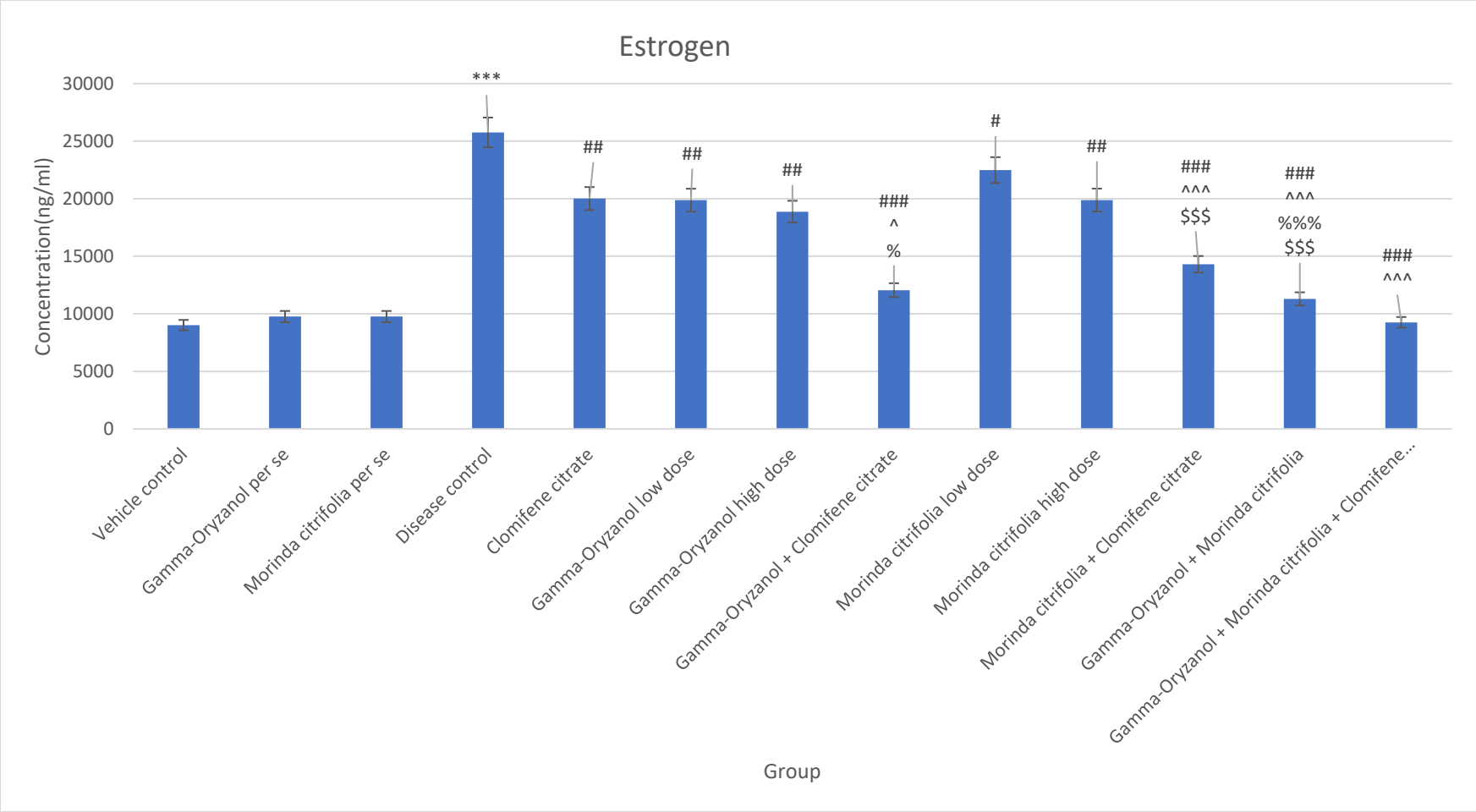


Fig 5.3.: Estrogen concentrations: The data are expressed as the mean values ± standard error of the mean (SEM) to give an indication of the variability and precision of the measurements. Statistical significance was evaluated based on the p-value thresholds: *p < 0.05 was considered statistically significant, **p < 0.01 indicated a highly significant difference, and *p < 0.001 denoted an extremely significant difference between the compared groups**."*** Comparison between control to *per se* and disease control group, **#** Comparison between disease control group to treatment groups, **^** Comparison between standard drug to treatment groups, **%** Comparison between gamma oryzanol treated groups to combination treatment groups (GO+CC and GO+MC), **$** Comparison between *Morinda citrifolia* treated groups to combination treatment groups (MC+CC and GO+MC), **@** Comparison between combination treatment groups (GO+MC *vs*. GO+MC+CC)

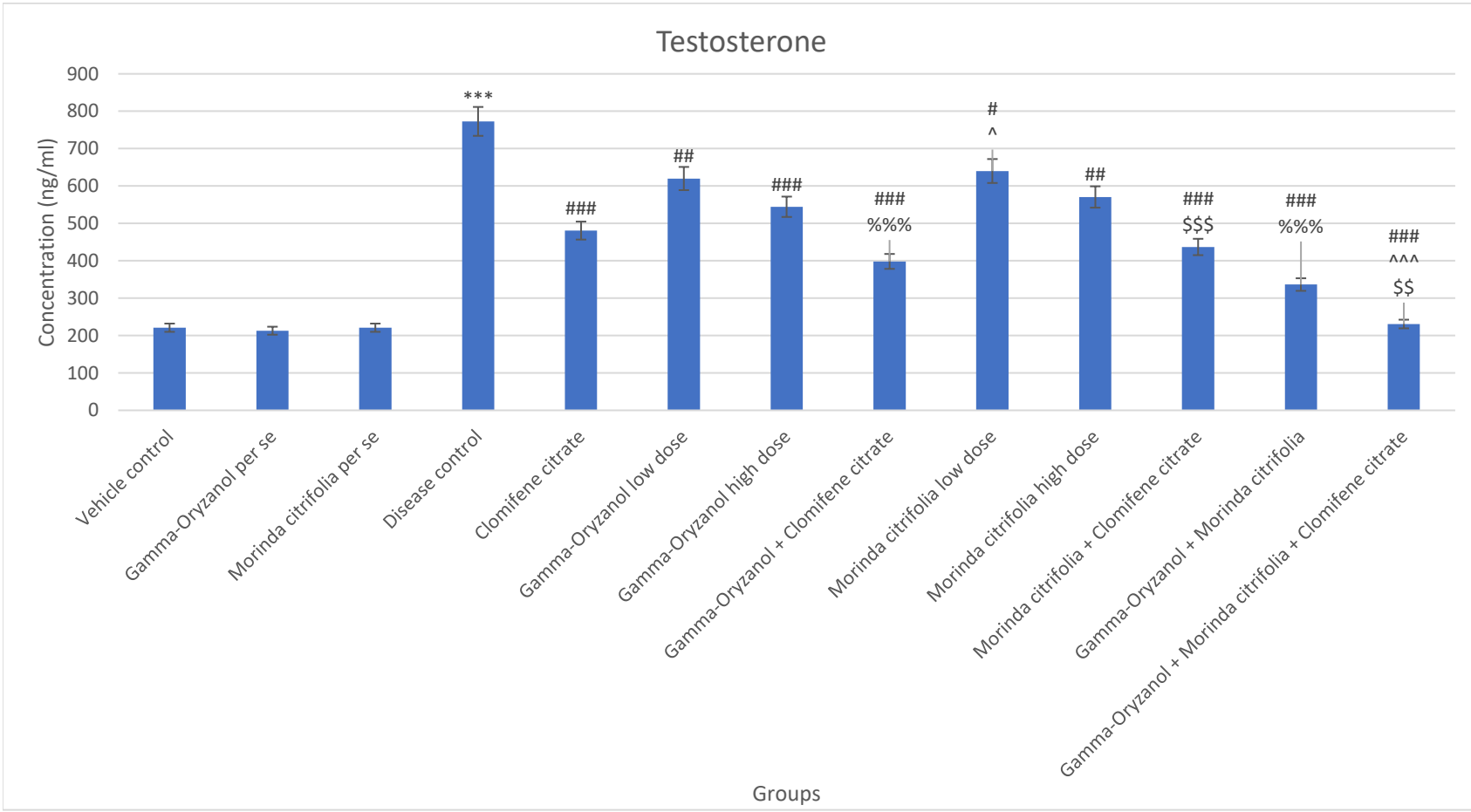


Fig 5.4.: Testosterone concentrations: The data are expressed as the mean values ± standard error of the mean (SEM) to give an indication of the variability and precision of the measurements. Statistical significance was evaluated based on the p-value thresholds: *p < 0.05 was considered statistically significant, **p < 0.01 indicated a highly significant difference, and *p < 0.001 denoted an extremely significant difference between the compared groups**."*** Comparison between control to *per se* and disease control group, # Comparison between disease control group to treatment groups, ^ Comparison between standard drug to treatment groups, **%** Comparison between gamma oryzanol treated groups to combination treatment groups (GO+CC and GO+MC), **$** Comparison between *Morinda citrifolia* treated groups to combination treatment groups (MC+CC and GO+MC), **@** Comparison between combination treatment groups (GO+MC *vs*. GO+MC+CC)

5.5. Enzymatic assay: The oxidative stress parameters were evaluated and the findings are as below.

5.6. Catalase activity (CAT): Statistically significant increase in the concentrations of CAT were assessed and observed in all of the different treatment groups., in contrast with the group that underwent treatment with estradiol valerate and for all the combination groups. Gamma oryzanol and Noni both exhibit significant increase in catalase level while compared with disease control group. There was a statistical a rise in the concentration of catalase in the group administered a treatment regimen consisting of both gamma oryzanol and clomifene citrate in comparison with the group that received the standard treatment regimen of clomifene citrate. There was a statistical a rise in the concentration of catalase in Noni and clomifene citrate combination treatment group in comparison with the group that received the standard treatment regimen of clomifene citrate. Catalase activity was decreased in Gamma-oryzanol + Noni + Clomifene citrate group while compared with Gamma-oryzanol + Noni but the decrease did not demonstrate statistical significance.

The concentration of catalase for the group receiving the combined treatment of gamma oryzanol and clomifene citrate shows 1.53-fold increase as compared to standard drug, similarly *Morinda citrifolia*+ Clomifene citrate group shows 1.6-fold increase while compared to standard drug.

The combination of Gamma oryzanol + *Morinda citrifolia* group shows 1.8-fold increase in concentration of catalase as compared to standard drug, similarly Gamma oryzanol+ *Morinda citrifolia*+ Clomifene citrate group shows 1.8-fold increase while compared to standard drug.

Table 5.4.: Serum concentrations of Catalase in groups

GROUP	CONCENTRATION (U CAT/mg protein)
Vehicle control	0.95±0.38
Gamma-oryzanol per se	0.94±0.38
Morinda citrifolia per se	0.91±0.37
Disease control	0.44±0.18***
Clomifene citrate	0.51±0.21#
Gamma-oryzanol (100 mg/kg p.o. low dose)	0.6±0.24#
Gamma-oryzanol (200 mg/kg p.o. high dose)	0.72±0.29#
Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate	0.78±0.32##^%
Morinda citrifolia (500 mg/kg p.o. low dose)	0.58±0.24#
Morinda citrifolia (1000 mg/kg p.o. high dose)	0.65±0.27#
Morinda citrifolia (500 mg/kg p.o. low dose) + Clomifene citrate	0.82±0.33#^$
Gamma-oryzanol + *Morinda citrifolia*	0.91±0.37###^^^%$$
Gamma-oryzanol + *Morinda citrifolia* (500 mg/kg p.o. low dose) + Clomifene citrate	0.92±0.38###^^^

5.7. Superoxide dismutase (SOD): Statistically significant increase in the concentrations of SOD were assessed and observed in all of the different treatment groups., in contrast with the group that underwent treatment with estradiol valerate and for all the combination groups. Gamma oryzanol and Noni both exhibit significant increase in SOD level while compared with disease control group. There was a statistical a rise in the concentration of SOD in the group administered a treatment regimen consisting of both gamma oryzanol and clomifene citrate in comparison with the group that received the standard treatment regimen of clomifene citrate. There was a statistical a rise in the concentration of SOD in Noni and clomifene citrate combination treatment group in comparison with the group that received the standard treatment regimen of clomifene citrate. SOD activity was decreased in Gamma-oryzanol + Noni + Clomifene citrate group while compared with Gamma-oryzanol + Noni but the decrease did not demonstrate statistical significance.

The SOD activity for the group receiving the combined treatment of gamma oryzanol and clomifene citrate shows 1.7-fold increase as compared to standard drug, similarly *Morinda citrifolia*+ Clomifene citrate group shows 1.6-fold increase while compared to standard drug.

The combination of Gamma oryzanol + *Morinda citrifolia* group shows 2.0-fold increase in SOD activity as compared to standard drug, similarly Gamma oryzanol+ *Morinda citrifolia*+ Clomifene citrate group shows 2.0-fold increase while compared to standard drug.

Table 5.5.: Serum concentrations of Superoxide dismutase in groups

GROUP	CONCENTRATION (U/g tissue)
Vehicle control	25±10.21
Gamma-oryzanol per se	24±9.80
Morinda citrifolia per se	24.5±10.00
Disease control	10.23±4.18***
Clomifene citrate	11.5±4.69#
Gamma-oryzanol (100 mg/kg p.o. low dose)	15.1±6.16#
Gamma-oryzanol (200 mg/kg p.o. high dose)	17±6.94#
Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate	19±7.76##^%
Morinda citrifolia (500 mg/kg p.o. low dose)	13.67±5.58#
Morinda citrifolia (1000 mg/kg p.o. high dose)	16±6.53##
Morinda citrifolia (500 mg/kg p.o. low dose) + Clomifene citrate	18±7.35#$
Gamma-oryzanol + *Morinda citrifolia*	22.4±9.14###^^^%$$
Gamma-oryzanol + *Morinda citrifolia* (500 mg/kg p.o. low dose) + Clomifene citrate	23.56±9.62###^^^

5.8. Reduced Glutathione activity (GSH): Statistically significant increase in concentrations of GSH were assessed and observed in all of the different treatment groups, in contrast with the group that underwent treatment with estradiol valerate and for all the combination groups. Gamma oryzanol and Noni both exhibit significant increase in GSH level while compared with disease control group. There was a statistical a rise in the concentration of GSH in the group administered a treatment regimen consisting of both gamma oryzanol and clomifene citrate in comparison with the group that received the standard treatment regimen of clomifene citrate. There was a statistical a rise in the concentration of GSH in Noni and clomifene citrate combination treatment group in comparison with the group that received the standard treatment regimen of clomifene citrate. GSH activity was decreased in Gamma-oryzanol + Noni + Clomifene citrate group while compared with Gamma-oryzanol + Noni but the decrease did not demonstrate statistical significance.

The GSH level for the group receiving the combined treatment of gamma oryzanol and clomifene citrate shows 1.4-fold increase as compared to standard drug, similarly *Morinda citrifolia*+ Clomifene citrate group shows 1.2-fold increase while compared to standard drug.

The combination of Gamma oryzanol + *Morinda citrifolia* group shows 1.7-fold increase in GSH level as compared to standard drug, similarly Gamma oryzanol+ *Morinda citrifolia*+ Clomifene citrate group shows 1.8-fold increase while compared to standard drug.

Table 5.6.: Serum concentrations of Reduced Glutathione in groups

GROUP	CONCENTRATION (nmol conjugated CDNB/min/mg protein)
Vehicle control	930±379.67
Gamma-oryzanol per se	950±387.84
Morinda citrifolia per se	945±385.79
Disease control	420±171.46***
Clomifene citrate	490±200.04#
Gamma-oryzanol (100 mg/kg p.o. low dose)	580±236.78##
Gamma-oryzanol (200 mg/kg p.o. high dose)	650±265.36##
Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate	690±281.69##^
Morinda citrifolia (500 mg/kg p.o. low dose)	560±228.62#
Morinda citrifolia (1000 mg/kg p.o. high dose)	600±244.95##
Morinda citrifolia (500 mg/kg p.o. low dose) + Clomifene citrate	610±249.03#
Gamma-oryzanol + *Morinda citrifolia*	840±342.93###^^^%$
Gamma-oryzanol + *Morinda citrifolia* (500 mg/kg p.o. low dose) + Clomifene citrate	920±375.59###^^^

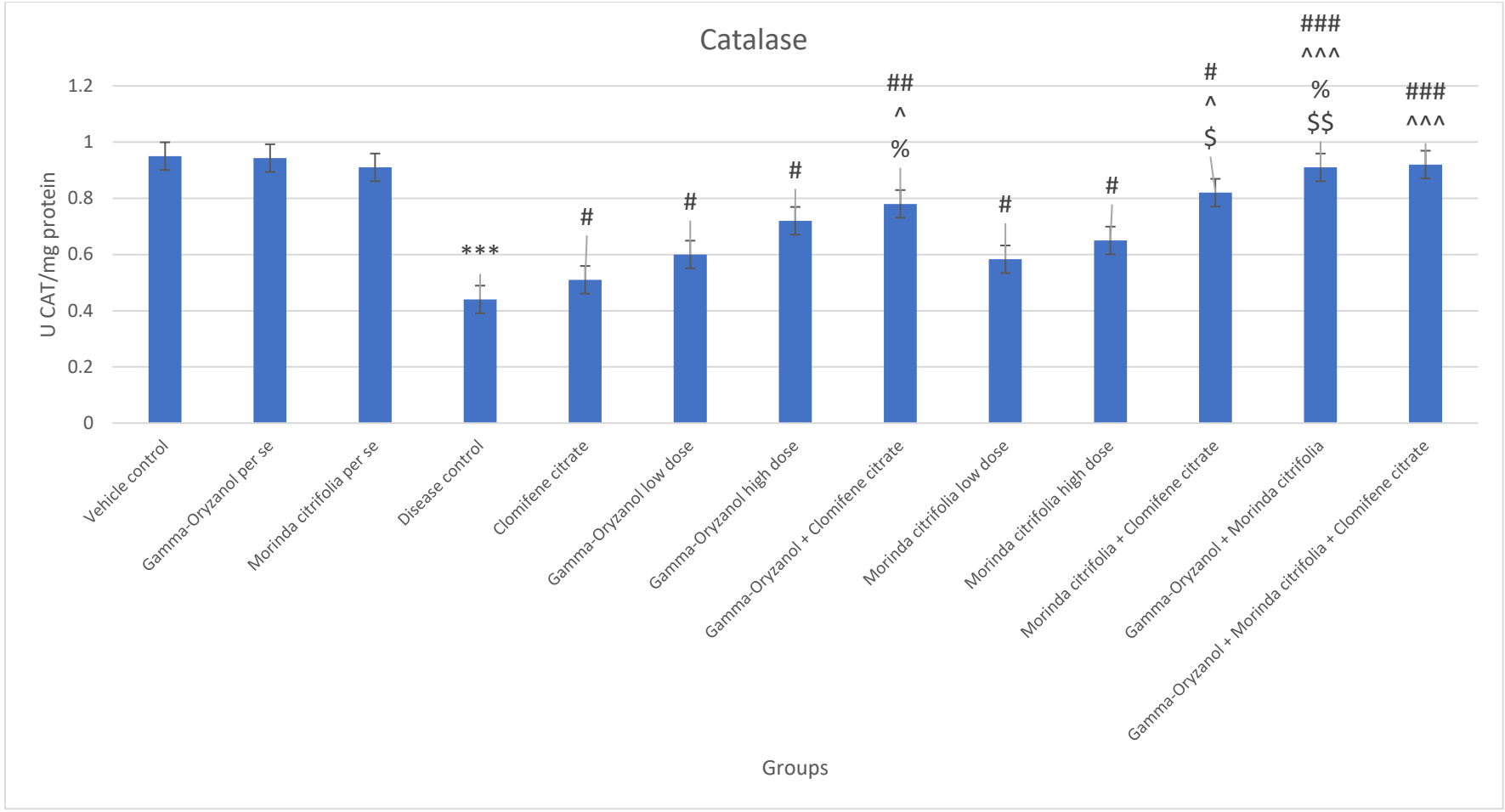


Fig 5.5.: CAT levels: The data are expressed as the mean values ± standard error of the mean (SEM) to give an indication of the variability and precision of the measurements. Statistical significance was evaluated based on the p-value thresholds: *p < 0.05 was considered statistically significant, **p < 0.01 indicated a highly significant difference, and *p < 0.001 denoted an extremely significant difference between the compared groups**."*** Comparison between control to *per se* and disease control group, **#** Comparison between disease control group to treatment groups, ^ Comparison between standard drug to treatment groups, **%** Comparison between gamma oryzanol treated groups to combination treatment groups (GO+CC and GO+MC), **$** Comparison between *Morinda citrifolia* treated groups to combination treatment groups (MC+CC and GO+MC), **@** Comparison between combination treatment groups (GO+MC *vs*. GO+MC+CC)

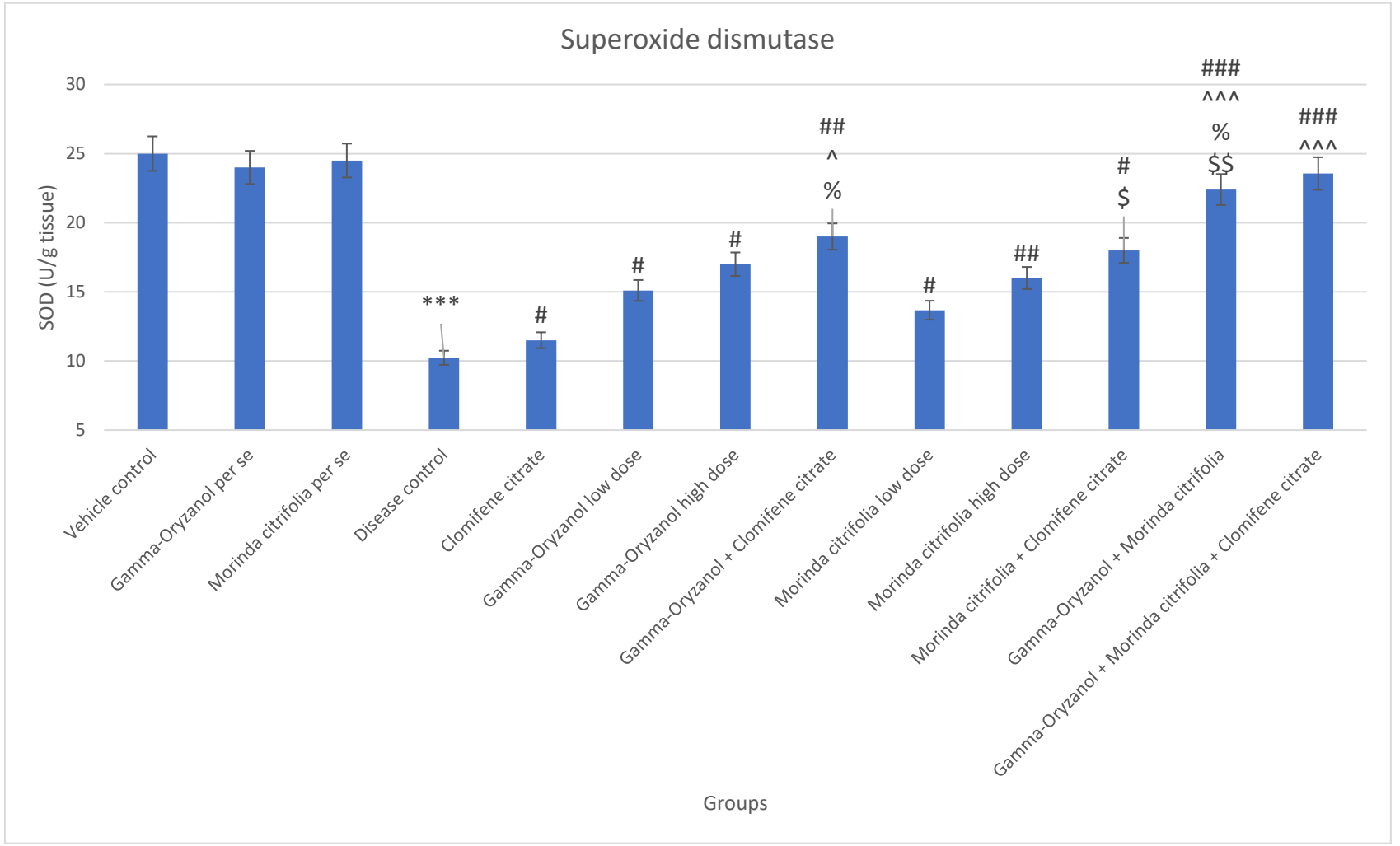


Fig 5.6.: SOD levels: The data are expressed as the mean values ± standard error of the mean (SEM) to give an indication of the variability and precision of the measurements. Statistical significance was evaluated based on the p-value thresholds: *p < 0.05 was considered statistically significant, **p < 0.01 indicated a highly significant difference, and *p < 0.001 denoted an extremely significant difference between the compared groups.**"*** Comparison between control to *per se* and disease control group, **#** Comparison between disease control group to treatment groups, **^** Comparison between standard drug to treatment groups, **%** Comparison between gamma oryzanol treated groups to combination treatment groups (GO+CC and GO+MC), **$** Comparison between *Morinda citrifolia* treated groups to combination treatment groups (MC+CC and GO+MC), **@** Comparison between combination treatment groups (GO+MC *vs*. GO+MC+CC)

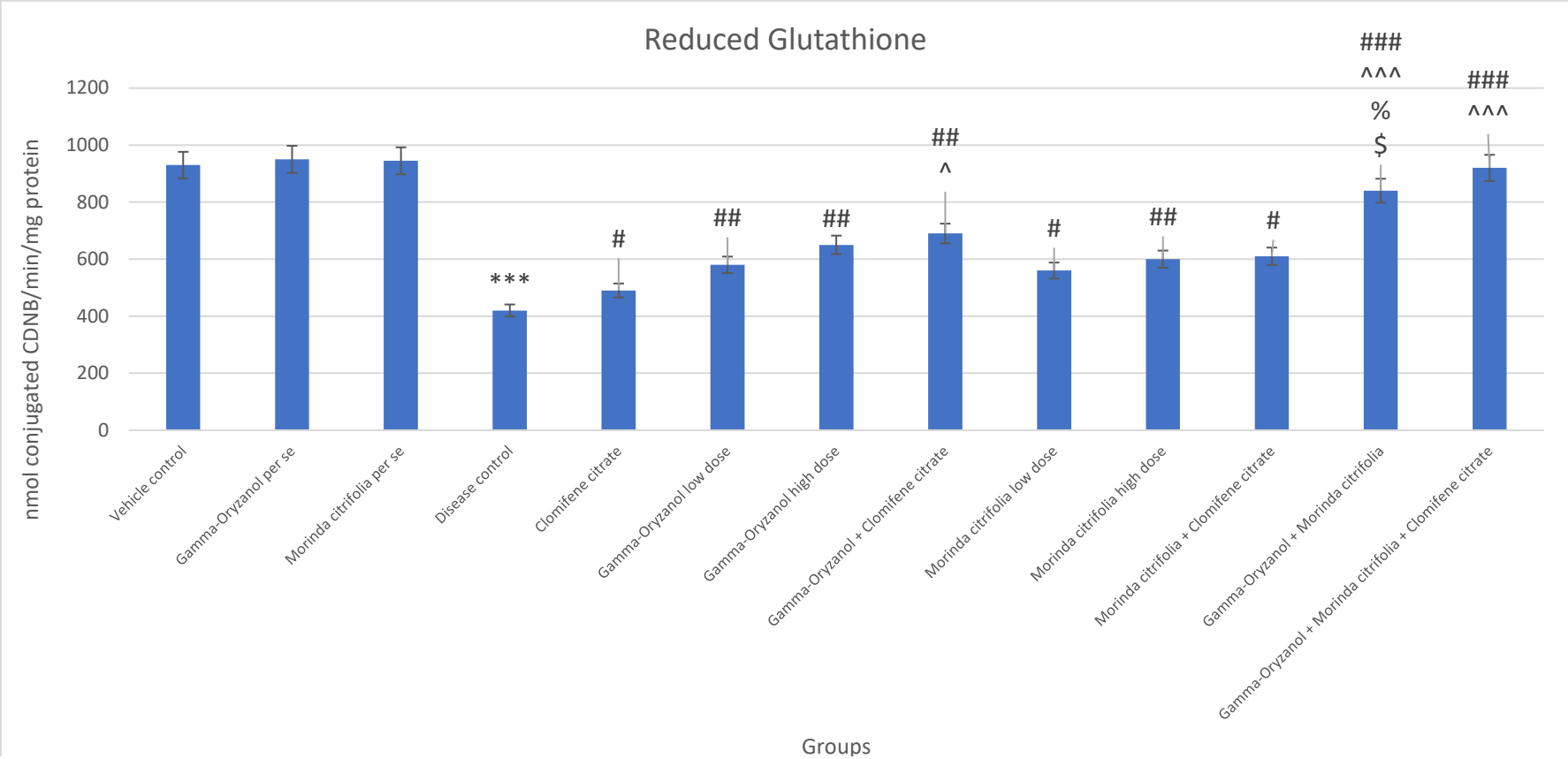


Fig 5.7.: GSH levels: The data are expressed as the mean values ± standard error of the mean (SEM) to give an indication of the variability and precision of the measurements. Statistical significance was evaluated based on the p-value thresholds: *p < 0.05 was considered statistically significant, **p < 0.01 indicated a highly significant difference, and *p < 0.001 denoted an extremely significant difference between the compared groups**."*** Comparison between control to *per se* and disease control group, # Comparison between disease control group to treatment groups, ^ Comparison between standard drug to treatment groups, **%** Comparison between gamma oryzanol treated groups to combination treatment groups (GO+CC and GO+MC), **$** Comparison between *Morinda citrifolia* treated groups to combination treatment groups (MC+CC and GO+MC), **@** Comparison between combination treatment groups (GO+MC *vs*. GO+MC+CC)

5.9. Body Weight

Statistically significant decrease in the wight of body were assessed and observed in all of the different treatment groups., in contrast with the group that underwent treatment with estradiol valerate and for all the combination groups.

The body weight for the group receiving the combined treatment of gamma oryzanol and clomifene citrate shows 1-fold decrease as compared to standard drug, similarly *Morinda citrifolia*+ Clomifene citrate group shows 1.05-fold increase while compared to standard drug.

The combination of Gamma oryzanol + *Morinda citrifolia* group shows 0.98-fold reduction in body weight compared to the standard treatment, similarly Gamma oryzanol+ *Morinda citrifolia*+ Clomifene citrate group shows 0.93-fold decrease while compared to standard drug.

Table 5.7.: Body weight in groups

GROUP	BODY WEIGHT (gms)
Vehicle control	250±102.06
Gamma-oryzanol per se	252±102.88
Morinda citrifolia per se	251±102.47
Disease control	380±155.13*
Clomifene citrate	280±114.31$^{\#\#\#}$
Gamma-oryzanol (100 mg/kg p.o. low dose)	310±126.56$^{\#\#}$
Gamma-oryzanol (200 mg/kg p.o. high dose)	305±124.52$^{\#\#}$
Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate	280±114.31$^{\#}$
Morinda citrifolia (500 mg/kg p.o. low dose)	334±136.35$^{\#}$
Morinda citrifolia (1000 mg/kg p.o. high dose)	325±132.68$^{\#}$
Morinda citrifolia (500 mg/kg p.o. low dose) + Clomifene citrate	295±120.43$^{\#}$
Gamma-oryzanol + *Morinda citrifolia*	275±112.27$^{\#\#\#}$
Gamma-oryzanol + *Morinda citrifolia* (500 mg/kg p.o. low dose) + Clomifene citrate	262±106.96$^{\#\#\#\$\$}$

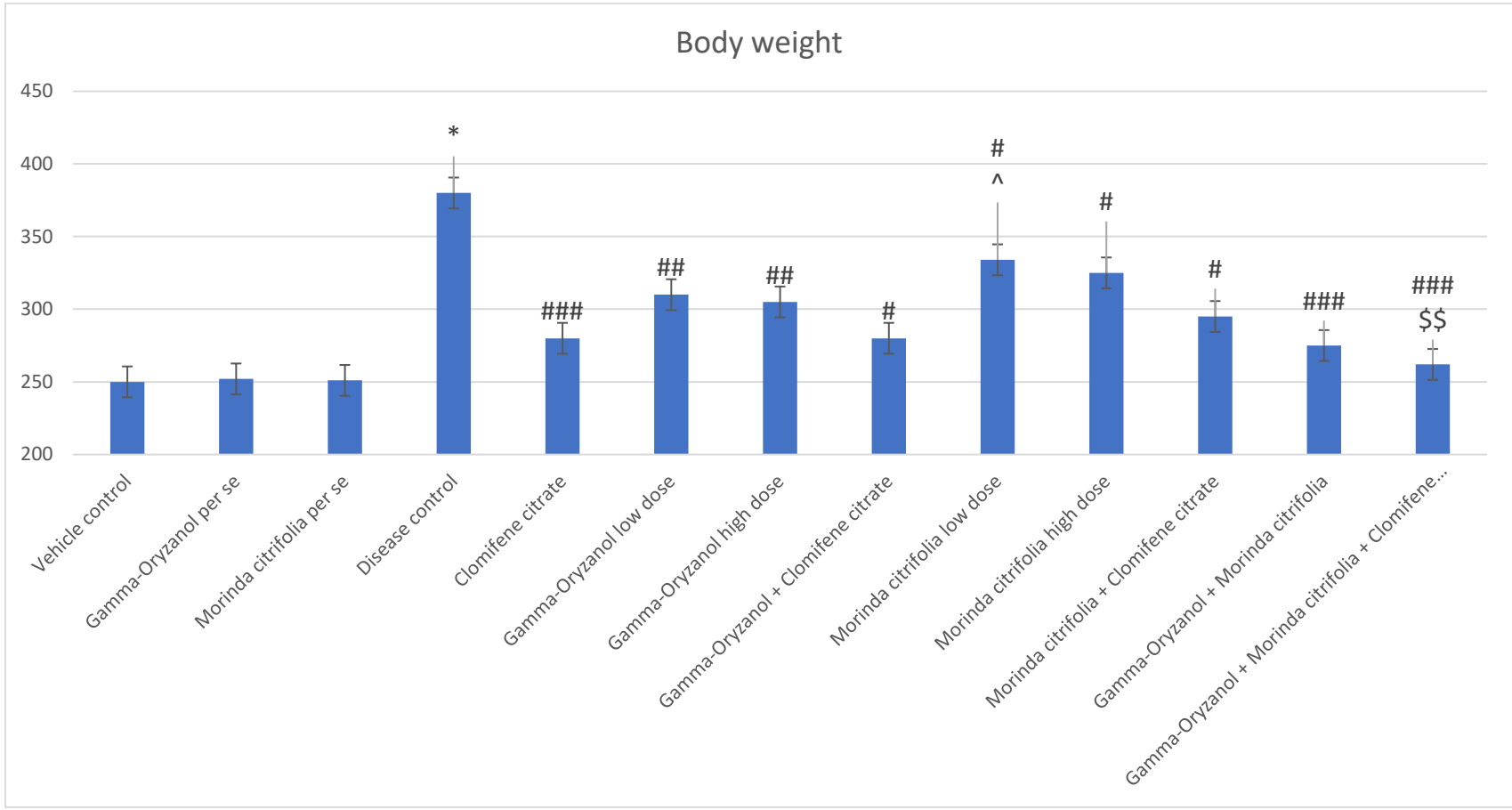


Fig 5.8.: Body weight: The data are expressed as the mean values ± standard error of the mean (SEM) to give an indication of the variability and precision of the measurements. Statistical significance was evaluated based on the p-value thresholds: *p < 0.05 was considered statistically significant, **p < 0.01 indicated a highly significant difference, and *p < 0.001 denoted an extremely significant difference between the compared groups**."*** Comparison between control to *per se* and disease control group, **#** Comparison between disease control group to treatment groups, ^ Comparison between standard drug to treatment groups, **%** Comparison between gamma oryzanol treated groups to combination treatment groups (GO+CC and GO+MC), **$** Comparison between *Morinda citrifolia* treated groups to combination treatment groups (MC+CC and GO+MC), **@** Comparison between combination treatment groups (GO+MC *vs*. GO+MC+CC)

5.10. Ovarian Weight

Statistically significant decrease in ovary weight assessed and observed in all of the different treatment groups., in contrast with the group that underwent treatment with estradiol valerate and for all the combination groups. Gamma oryzanol and Noni both exhibit significant decrease in ovarian weight while compared with disease control group. Decrease in ovarian weight was decreased in Gamma-oryzanol + Noni + Clomifene citrate group while compared with Gamma-oryzanol + Noni and it was statistically significant.

The ovarian weight for the group receiving the combined treatment of gamma oryzanol and clomifene citrate shows 0.98-fold decrease as compared to standard drug, similarly *Morinda citrifolia*+ Clomifene citrate group shows 0.96-fold decrease while compared to standard drug.

The combination of Gamma oryzanol + *Morinda citrifolia* group shows 0.98-fold rreduction in body weight compared to the standard treatment, similarly Gamma oryzanol+ *Morinda citrifolia*+ Clomifene citrate group shows 0.88-fold decrease while compared to standard drug.

Table 5.8.: Ovarian weight in groups

GROUPS	OVARIAN WEIGHT (gms)
control	0.04±0.02
Gamma-oryzanol per se	0.04±0.02
Morinda citrifolia per se	0.04±0.03
Disease control	0.08±0.02***
Clomifene citrate	0.05±0.03###
Gamma-oryzanol (100 mg/kg p.o. low dose)	0.06±0.02#
Gamma-oryzanol (200 mg/kg p.o. high dose)	0.06±0.02#
Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate	0.05±0.03###%
Morinda citrifolia (500 mg/kg p.o. low dose)	0.07±0.03#
Morinda citrifolia (1000 mg/kg p.o. high dose)	0.06±0.02##^
Morinda citrifolia (500 mg/kg p.o. low dose) + Clomifene citrate	0.05±###$
Gamma-oryzanol + *Morinda citrifolia*	0.05±0.02###%$
Gamma-oryzanol + *Morinda citrifolia* (500 mg/kg p.o. low dose) + Clomifene citrate	0.04±0.02###^

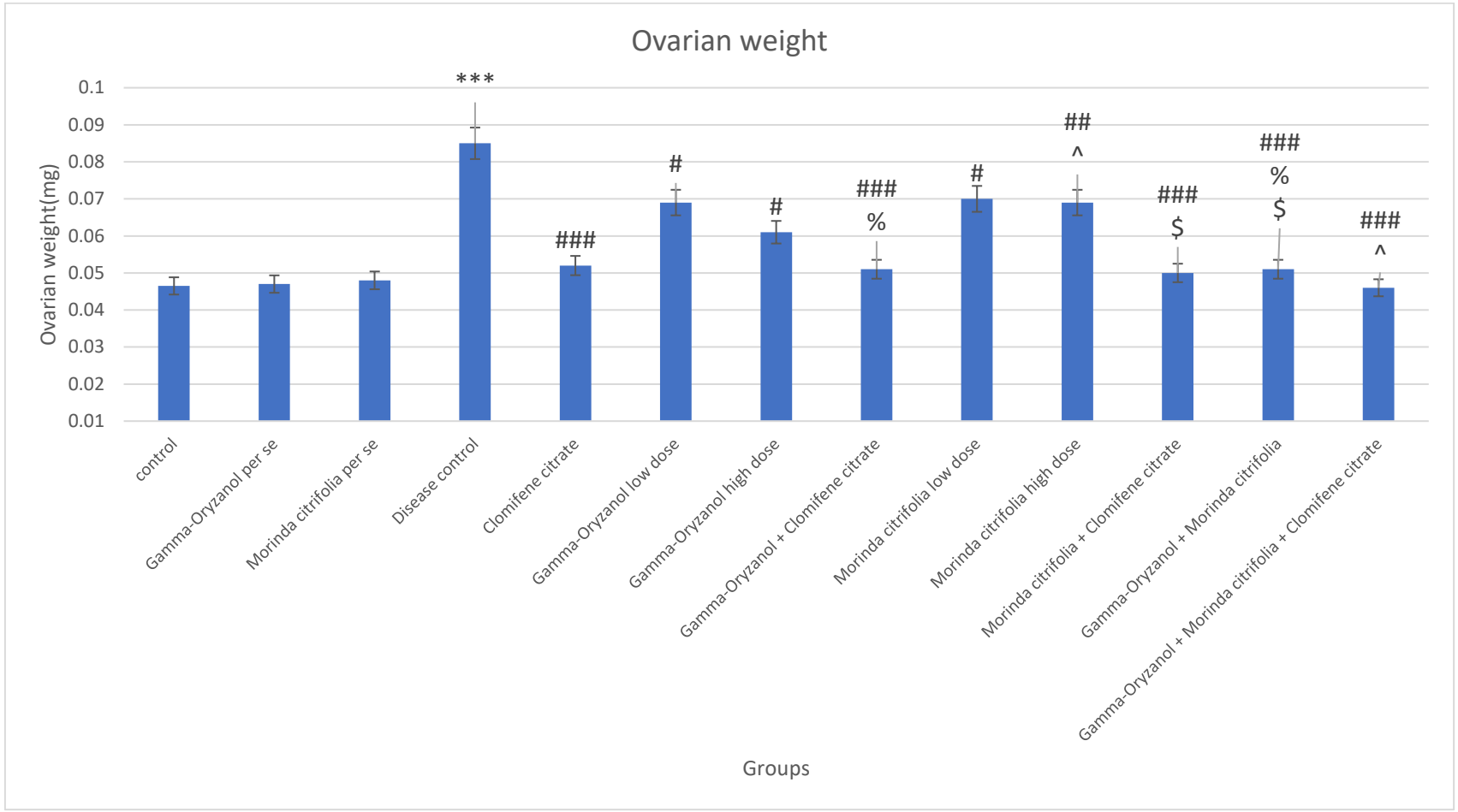


Fig 5.9.: Ovarian weight: The data are expressed as the mean values ± standard error of the mean (SEM) to give an indication of the variability and precision of the measurements. Statistical significance was evaluated based on the p-value thresholds: *p < 0.05 was considered statistically significant, **p < 0.01 indicated a highly significant difference, and *p < 0.001 denoted an extremely significant difference between the compared groups**."*** Comparison between control to *per se* and disease control group, **#** Comparison between disease control group to treatment groups, ^ Comparison between standard drug to treatment groups, **%** Comparison between gamma oryzanol treated groups to combination treatment groups (GO+CC and GO+MC), **$** Comparison between *Morinda citrifolia* treated groups to combination treatment groups (MC+CC and GO+MC), **@** Comparison between combination treatment groups (GO+MC *vs*. GO+MC+CC)

5.11. Fertility Study

Fertility rate for the vehicle control, Gamma oryzanol per se, *Morinda citrifolia* per se group did not shows any changes. The disease control depicts a statistically significant increase in fertility rate. The standard drug clomifene citrate, gamma oryzanol (Both doses) and *Morinda citrifolia* (Both doses) groups shows a significant upsurge in fertility rate in contrast to the disease control group. gamma oryzanol (Both doses) and *Morinda citrifolia* (Both doses) groups depicts a statistically significant improvement in fertility rate while compared with standard drug. Gamma oryzanol+ Clomifene citrate and *Morinda citrifolia*+ Clomifene citrate group depicts a statistically significant increase in fertility rate while compared with only test drug (both dose) groups. The group receiving the combined treatment of gamma oryzanol and clomifene citrate also shows an increase in fertility rate as compared with standard drug. Gamma oryzanol+ *Morinda citrifolia* group depicts a statistically significant increase in fertility rate in comparison with disease group, Gamma oryzanol and *Morinda citrifolia* both dose groups. Gamma oryzanol+ *Morinda citrifolia*+ Clomifene citrate group depicts a statistically significant increase in fertility rate in comparison with disease control standard drug group and also in comparison with combination groups (GO+MC).

The fertility rate for the group receiving the combined treatment of gamma oryzanol and clomifene citrate shows 1.17-fold increase as compared to standard drug, similarly *Morinda citrifolia*+ Clomifene citrate group shows 1-fold increase while compared to standard drug.

Table 5.9.: Number of implantation sites in groups

GROUPS	NO OF IMPLANTATION SITES
Vehicle control	10±4.08
Gamma-oryzanol per se	12±4.90
Morinda citrifolia per se	11±4.49
Disease control	0±0.00***
Clomifene citrate	6±2.45###
Gamma-oryzanol (100 mg/kg p.o. low dose)	1±0.41#^^^
Gamma-oryzanol (200 mg/kg p.o. high dose)	2±0.82##^^^
Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate	7±2.86###^%%%
Morinda citrifolia (500 mg/kg p.o. low dose)	0±0.00^^^
Morinda citrifolia (1000 mg/kg p.o. high dose)	1±0.41^^^
Morinda citrifolia (500 mg/kg p.o. low dose) + Clomifene citrate	6±2.45###$$$
Gamma-oryzanol + *Morinda citrifolia*	4±1.63###%%%$$$
Gamma-oryzanol + *Morinda citrifolia* (500 mg/kg p.o. low dose) + Clomifene citrate	9±3.67###^^^@@@

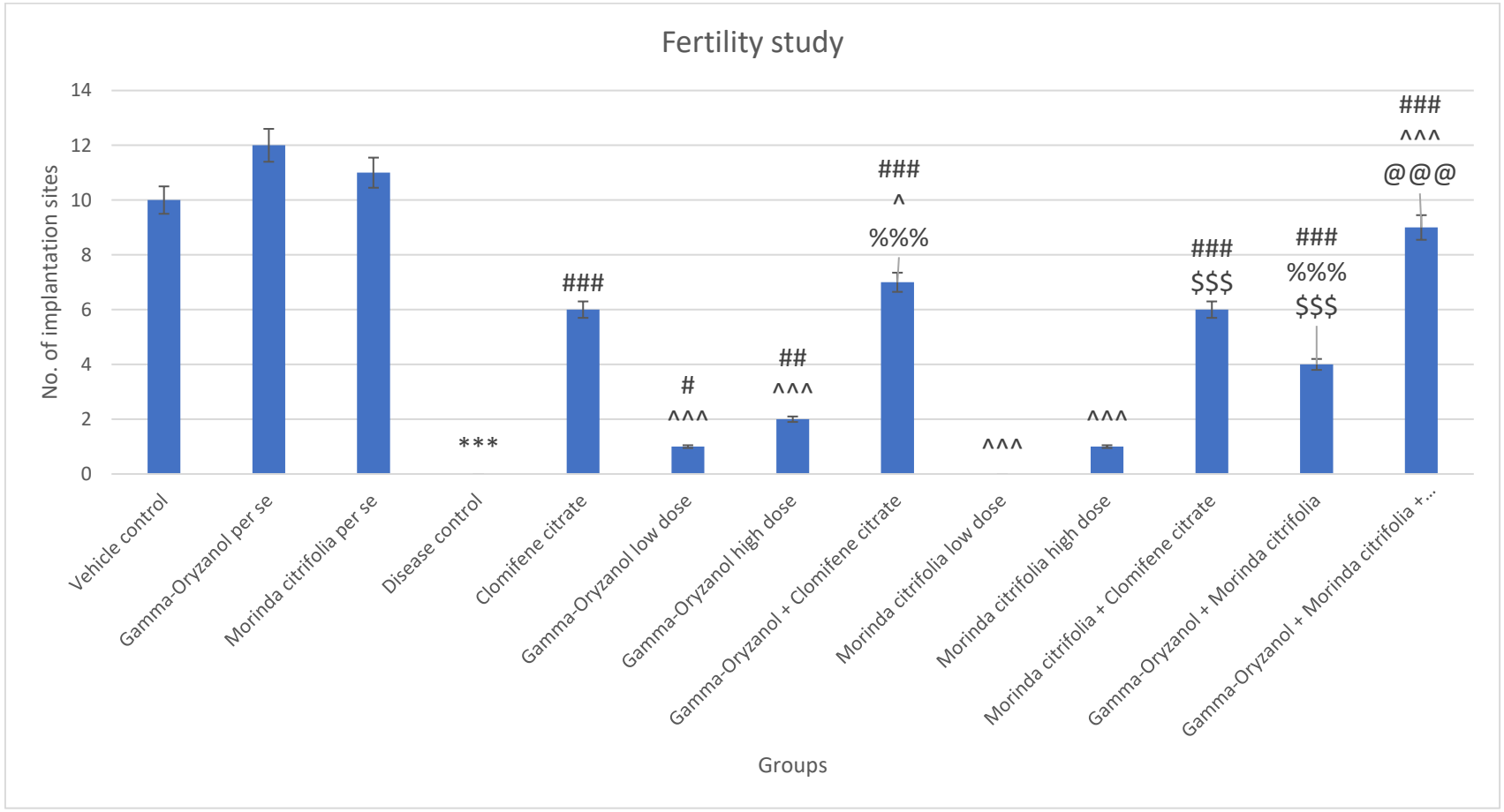


Fig 5.10.: Fertility Study (no of implantation sites): The data are expressed as the mean values ± standard error of the mean (SEM) to give an indication of the variability and precision of the measurements. Statistical significance was evaluated based on the p-value thresholds: *p < 0.05 was considered statistically significant, **p < 0.01 indicated a highly significant difference, and *p < 0.001 denoted an extremely significant difference between the compared groups.**"*** Comparison between control to *per se* and disease control group, **#** Comparison between disease control group to treatment groups, **^** Comparison between standard drug to treatment groups, **%** Comparison between gamma oryzanol treated groups to combination treatment groups (GO+CC and GO+MC), **$** Comparison between *Morinda citrifolia* treated groups to combination treatment groups (MC+CC and GO+MC), **@** Comparison between combination treatment groups (GO+MC *vs*. GO+MC+CC)

5.12. Histopathological Analysis

Histopathological examination of the group treated with estradiol valerate revealed the presence of multiple cystic formations within the ovaries, indicating significant disruption in ovarian structure. Follicular maturation was arrested at various stages, leading to the development of polycystic ovarian morphology, a condition commonly associated with hormonal imbalances. In contrast, in the treatment groups, this disruption appeared to be reversed to varying extents, with noticeable improvements in ovarian architecture. Notably, in all combination treatment groups, a decrease in the number of follicles was observed, suggesting a potential alteration in follicular dynamics as a result of the combined therapies (Fig 11)

Table 5.10.: Histopathological findings

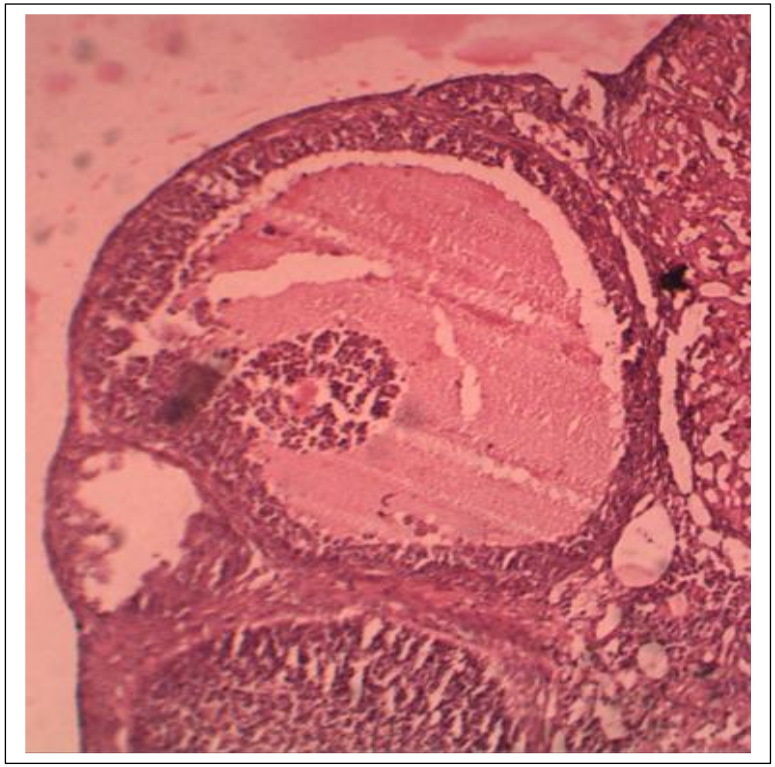
GROUPS	HISTOPATHOLOGICAL FINDINGS
Group 1: Vehicle control	Normal architecture of ovary is observed. A healthy advanced follicle is present.
Group 2: Gamma-oryzanol *per se* treated group	Normal architecture of ovary is observed. A healthy advanced follicle is present.
Group 3: *Morinda citrifolia per se* treated group	Normal architecture of ovary is observed. A healthy advanced follicle is present.
Group 4: Disease control group	Normal architecture of ovary is not preserved. No specific healthy follicle is observed. Multiple cystic structure is observed, which are arrested at various stages of maturation. Disruption of overall cellular structure is observed. The central and peripheral stroma both are disrupted.
Group 5: Clomifene citrate treated group	The disruption is central and peripheral stroma is much better than G4. Multiple cysts are also present. One

	healthy mature follicle is present and one follicle in early maturation stage can be observed. A corpus luteum is also present.
Group 6: Gamma-oryzanol (100 mg/kg p.o. low dose) treated group	The disruption is central and peripheral stroma is not significantly improved as compared to G4. Multiple cysts are still present. One follicle in early maturation stage can be observed.
Group 7: Gamma-oryzanol (200 mg/kg p.o. high dose) treated group	The disruption is central and peripheral stroma is improved as compared to G4. Multiple cysts are not present. No healthy mature follicle is present.
Group 8: Gamma-oryzanol (100 mg/kg p.o. low dose) + Clomifene citrate treated group	The disruption is central and peripheral stroma is significantly improved as compared to G4. Multiple cysts are not visible. Two follicles in early maturation stage can be observed.
Group 9: *Morinda citrifolia (500 mg/kg p.o. low dose)* treated group	The disruption is central and peripheral stroma is not significantly improved as compared to G4. Multiple cysts are not visible. No healthy mature follicle is present. A corpus luteum is present.
Group 10: *Morinda citrifolia (1000 mg/kg p.o. high dose)* treated group	The disruption is central and peripheral stroma is not significantly improved as compared to G4. Multiple cysts are not visible. No healthy mature follicle is present. A corpus luteum is present.
Group 11: *Morinda citrifolia (500 mg/kg p.o. low dose) + Clomifene citrate* drug treated group)	The disruption is central and peripheral stroma is significantly improved as compared to G4. Multiple cysts are not visible. No healthy follicle and no corpus luteum is present.

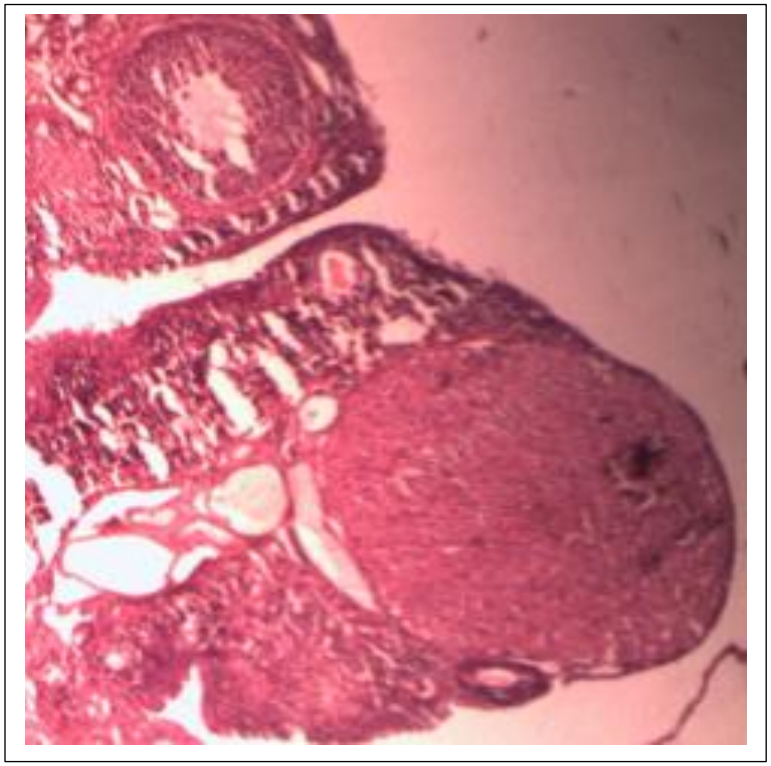
Group 12: Gamma-oryzanol + *Morinda citrifolia* drug treated group)	The disruption is central and peripheral stroma is improved as compared to G4. Multiple cysts are not visible. No healthy follicle is observed. One corpus luteum is present.
Group 13: Gamma-oryzanol + *Morinda citrifolia* + Clomifene citrate drug treated group	The disruption is central and peripheral stroma is significantly improved as compared to G4. Multiple cysts are not visible. An advanced healthy follicle is observed. No corpus luteum is present.

As per the above table it was observed that the disruption of cellular architecture was greater in the G4; disease control group. Multiple cysts were there in G4. In the treatment groups, disruption of cellular architecture was restored, instead of multiple cysts either a healthy mature follicle or a follicle in its early maturation stage was observed. We also able to observe a corpus luteum in various groups which indicates a successful ovulation. Hence these findings indicates that Gamma-oryzanol and *Morinda citrifolia* provide an adjuvant therapy in PCOS condition.

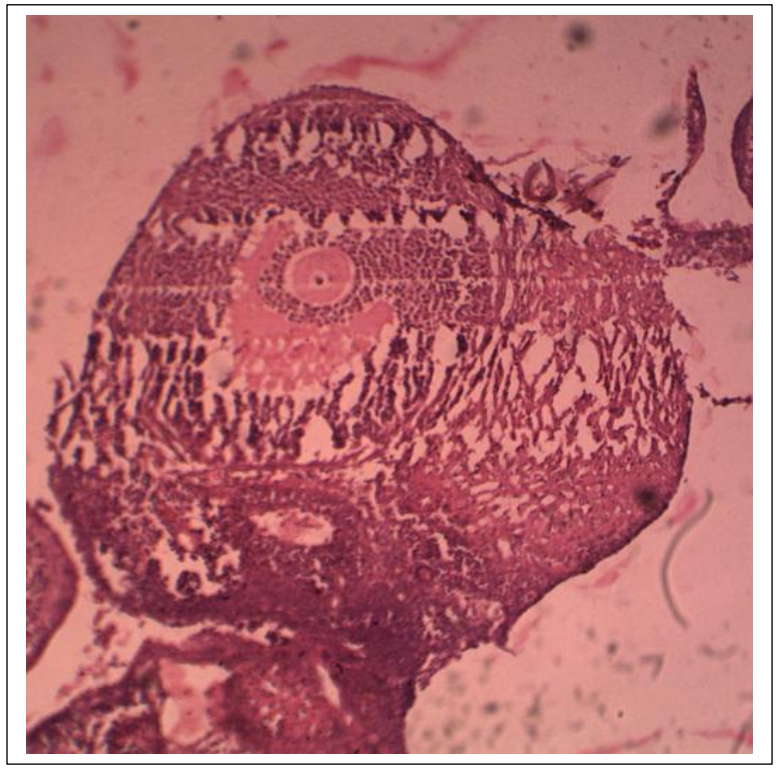
5.7. Histopathology



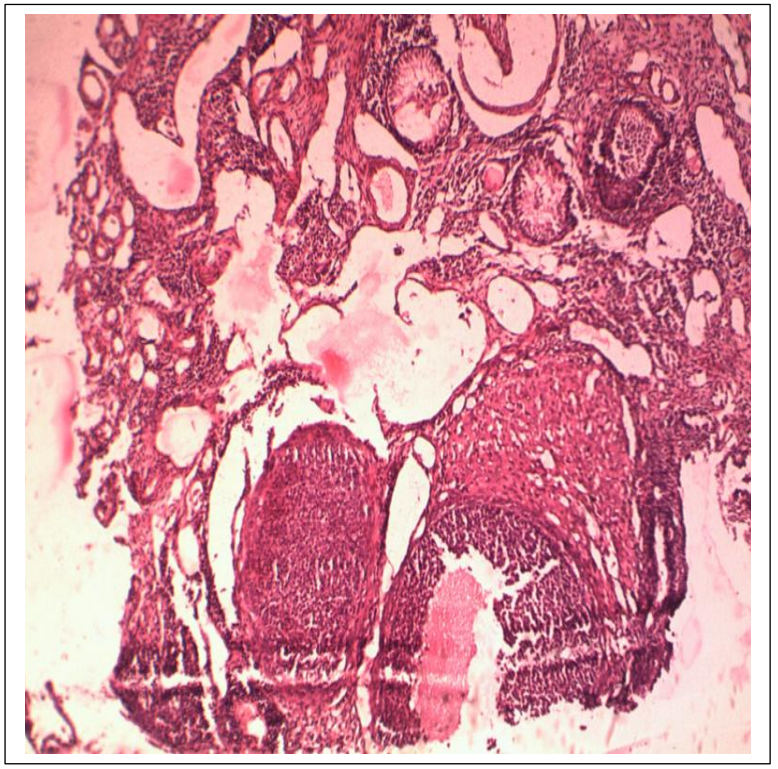
5.12. Control



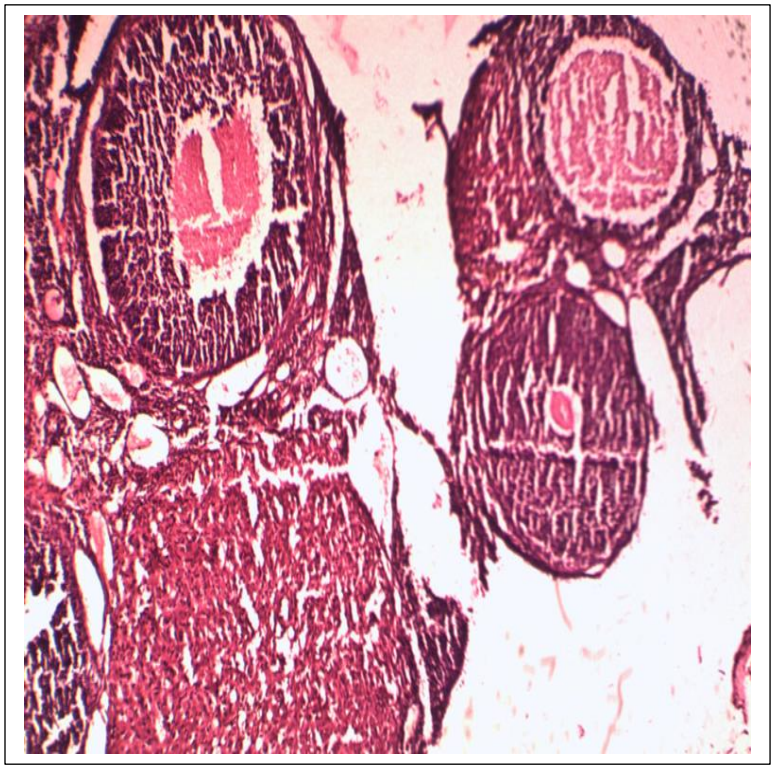
5.13. Gamma-Oryzanol per se



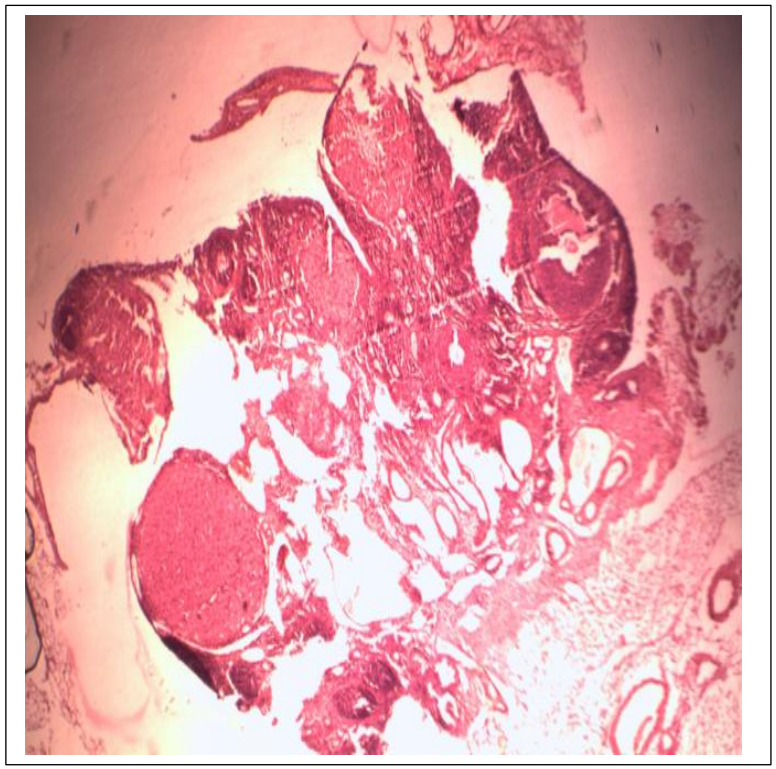
5.14. *Morinda citrifolia* per se



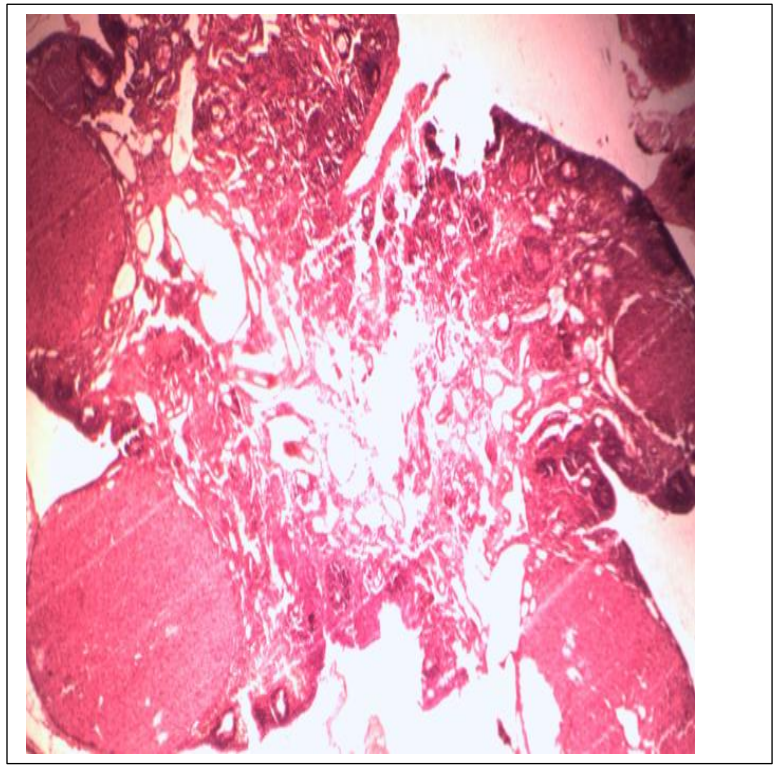
5.15. Disease control



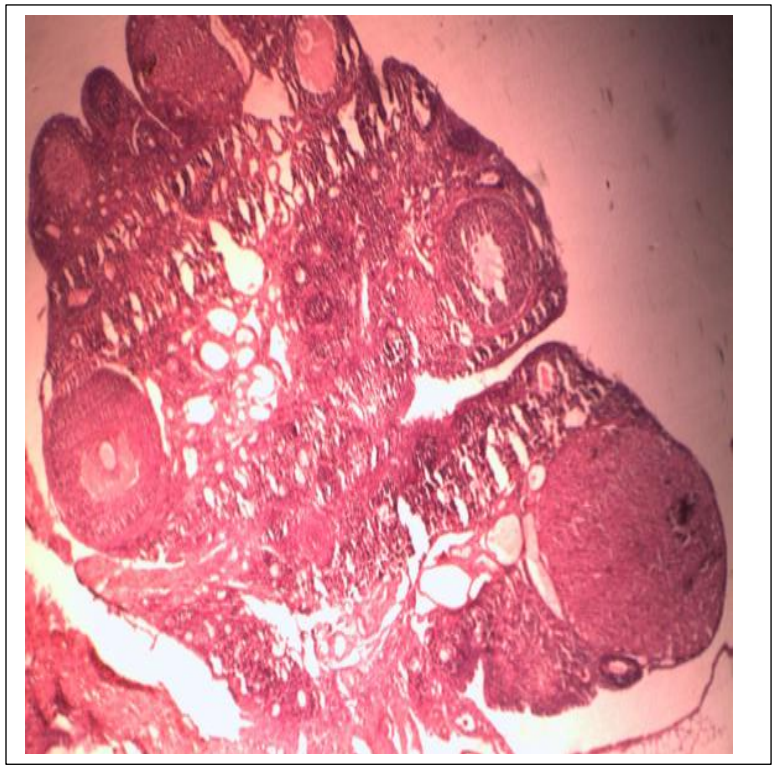
5.16. Clomifene citrate



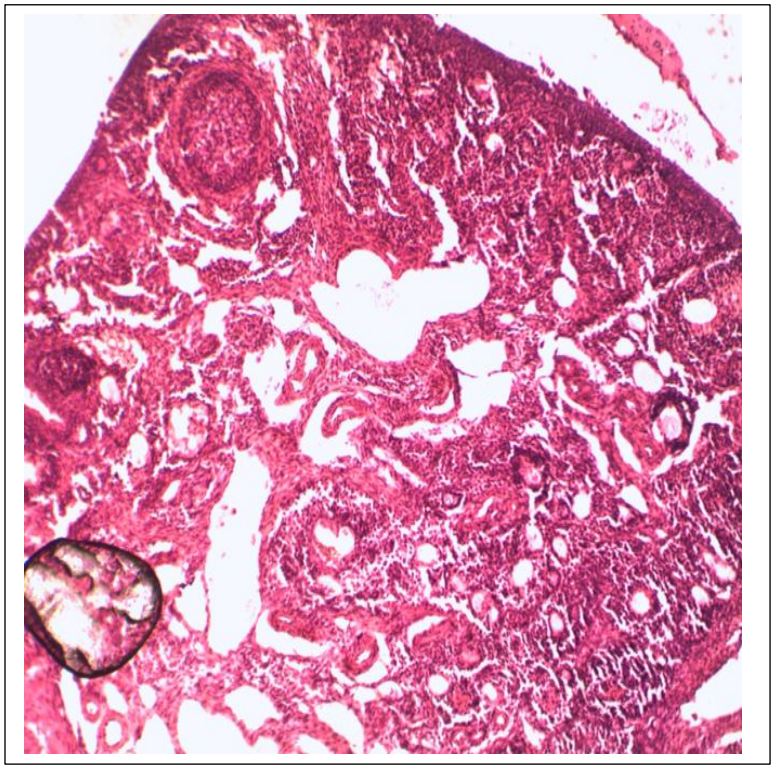
5.17. Gamma-Oryzanol low dose



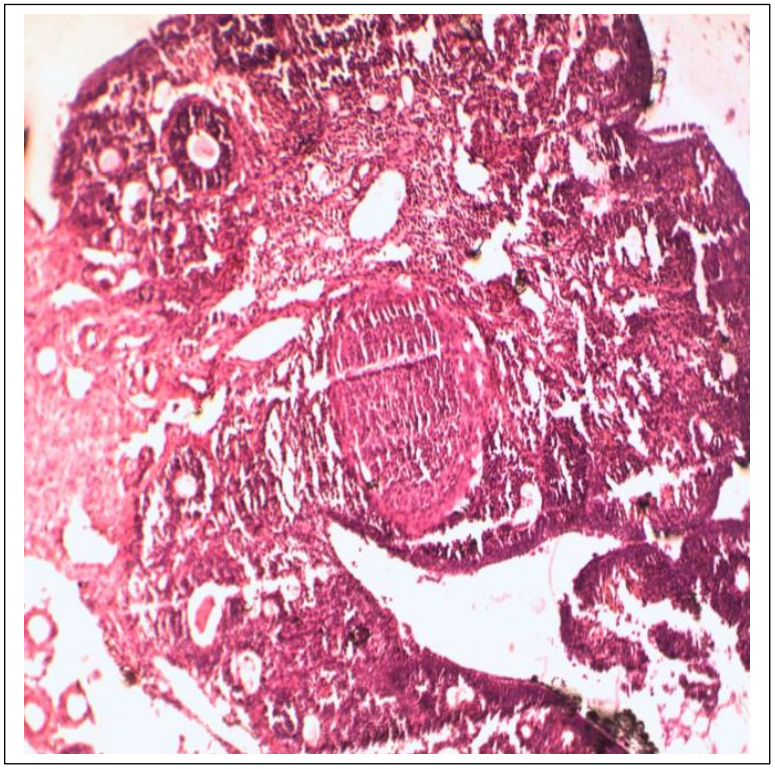
5.18. Gamma-Oryzanol high dose



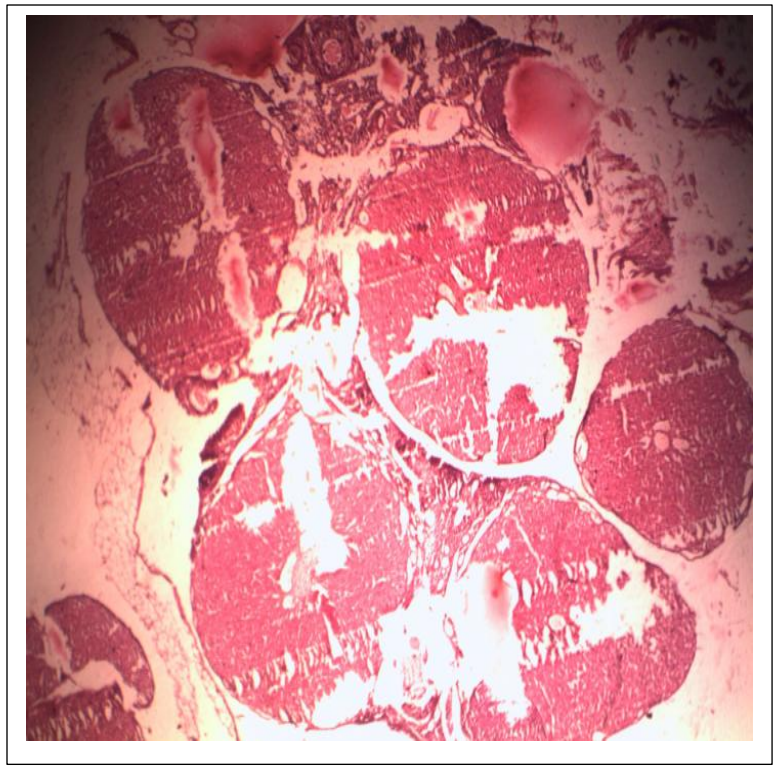
5.19. Gamma-Oryzanol + Clomifene citrate



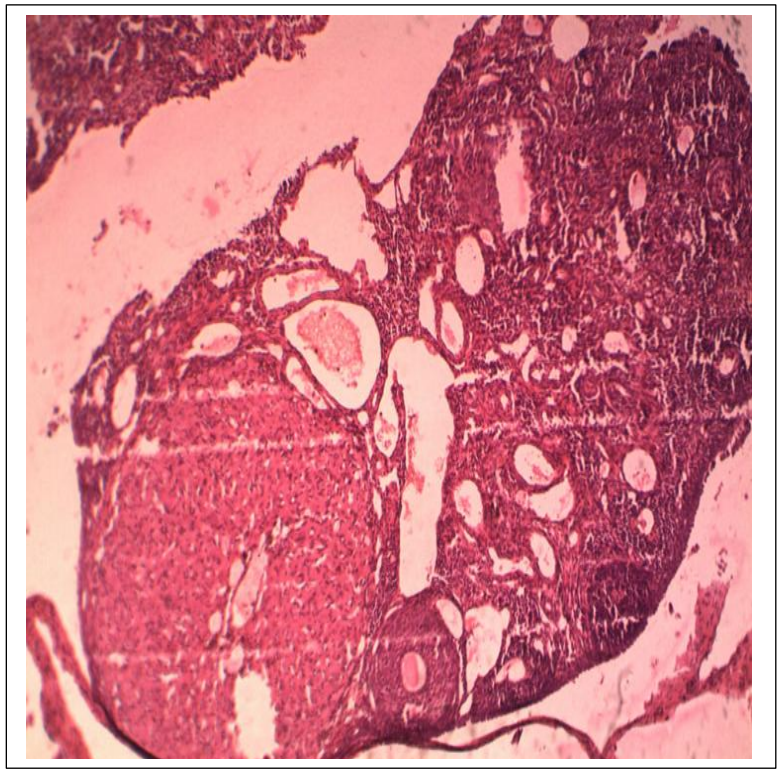
5.20. *Morinda citrifolia* low dose



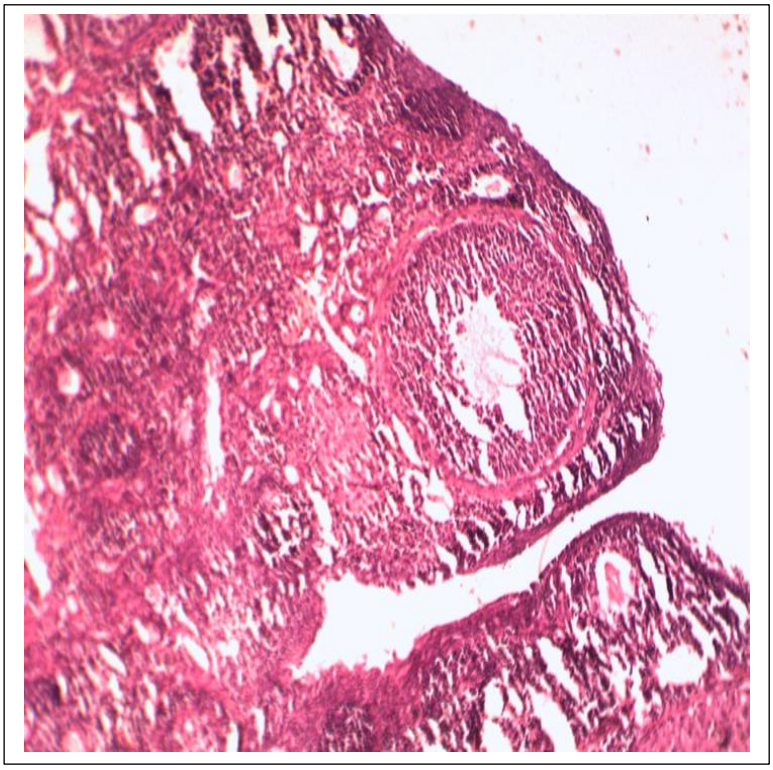
5.21. *Morinda citrifolia* high dose



5.22. *Morinda citrifolia* + Clomifene citrate



5.23. Gamma-Oryzanol + *Morinda citrifolia*



5.24. Gamma-Oryzanol + *Morinda citrifolia* + Clomifene citrate

Fig 5.11.: Histopathological images of overies in different groups

Discussion

Polycystic ovary syndrome (PCOS) is a multifactorial and highly heterogeneous endocrine disorder, involving a diverse range of hormonal imbalances and irregularities. These hormonal disruptions can lead to a variety of interconnected health problems, manifesting as a combination of reproductive, metabolic, and psychological disturbances. As a result, women with PCOS may experience a wide spectrum of symptoms, affecting not only their reproductive health, such as irregular menstrual cycles and infertility, but also contributing to metabolic issues like insulin resistance and obesity, as well as mental health challenges such as anxiety and depression. This condition is predominantly governed by symptoms of hyperandrogenism, which refers to elevated levels of male sex hormones, including androgen and testosterone, resulting in noticeable signs such as hirsutism (excessive body hair), acne, and scalp hair thinning. Women with PCOS often experience irregular or absent menstrual cycles, which result from disrupted ovarian function and hormonal imbalances. These fluctuations in hormones, particularly elevated levels of androgens, can interfere with the normal ovulation process, leading to infrequent or missed periods (Singh et al., 2023). As a consequence, women with PCOS may face challenges related to fertility and reproductive health. Along with these menstrual abnormalities, the condition is often marked by the presence of multiple cystic structures within the ovaries, which arise due to incomplete follicular maturation. As a result, the ovaries may become enlarged, contributing to the overall increase in ovarian weight. In addition to these reproductive issues, PCOS is strongly associated with infertility, as the hormonal imbalances interfere with normal ovulation. Metabolically, women with PCOS are at a significantly higher risk of developing various conditions such as obesity, insulin resistance, and eventually type 2 diabetes (Rubin et al., 2017). These metabolic abnormalities, which often occur in tandem with the hormonal imbalances' characteristic of PCOS, further complicate the management and treatment of the condition. The presence of insulin resistance, for instance, can lead to weight gain and difficulties with weight management, while also increasing the risk of developing more serious long-term health problems like cardiovascular disease. Together, these interconnected metabolic issues create a complex clinical picture, requiring a multifaceted approach to treatment and ongoing monitoring.

Together, these symptoms and metabolic disturbances form the clinical hallmark of PCOS, making it a complex disorder that requires a comprehensive approach for diagnosis and treatment (Teede et al., 2010). Currently, the "Rotterdam Criteria" is the standard diagnostic tool used to confirm the diagnosis of polycystic ovary syndrome (PCOS) in patients. This set of diagnostic criteria is based on three key conditions: the presence of anovulation or oligovulation (irregular or absent ovulation), signs of hyperandrogenism (condition characterized by an excessive production or presence of androgens (male hormones, such as testosterone) in the body, particularly in individuals assigned female at birth. This hormonal imbalance can lead to a range of symptoms, including excessive facial and body hair (hirsutism), acne, thinning hair or male-pattern baldness, and irregular menstrual cycles), and the exclusion of other conditions with similar presentations, such as Cushing's syndrome, androgen-secreting tumors, or adrenal hyperplasia (Christ & Cedars, 2023). For a PCOS diagnosis to be confirmed, a patient must meet at least two of these three criteria. The presence of two or more of these features, after ruling out other potential causes, establishes the diagnosis of PCOS, making the Rotterdam Criteria a crucial tool for clinicians in identifying the condition. (Kamenov & Gateva, 2020).

Recent studies have indicated that women with polycystic ovary syndrome (PCOS), particularly those who also have obesity and elevated androgen levels, are at a significantly higher risk for developing a range of serious health complications. These include an increased susceptibility to type 2 diabetes due to insulin resistance, a hallmark feature of PCOS. Moreover, this combination of obesity and hormonal imbalance further heightens the risk of developing cardiovascular diseases, including potentially life-threatening conditions such as stroke and coronary heart disease (Hoeger et al., 2021). The presence of these metabolic and hormonal factors creates a dangerous synergistic effect, making women with PCOS more vulnerable to long-term health issues, which underscores the importance of early diagnosis and comprehensive management of the condition. (Zhu et al., 2020).

Polycystic ovary syndrome (PCOS) is primarily considered a metabolic disorder, meaning it involves multiple biochemical and hormonal irregularities that disrupt the body’s normal functioning. Due to its complex nature, PCOS cannot currently be fully cured, as there is no single treatment that addresses all of its underlying causes. The disorder is characterized by a combination of various signs and symptoms, including menstrual irregularities, ovarian cysts,

hyperandrogenism, and metabolic disturbances such as insulin resistance, which makes it a multifaceted condition that is often challenging to manage. While a definitive cure for PCOS remains unavailable at present, multiple treatment options exist to ease symptoms and boost quality of life. These approaches may include medications to regulate menstrual cycles, reduce androgen levels, and manage metabolic issues, along with lifestyle modifications to address weight gain and insulin resistance. Furthermore, certain treatments can provide women with effective contraception and symptomatic relief, helping them manage the condition and reduce the risk of complications such as infertility, diabetes, and cardiovascular diseases (Collée et al., 2021)(Khan et al., 2019). A daily intake of 1500 calories through a low-fat diet, when combined with the use of metformin, has been shown to be an effective strategy for managing and reducing the symptoms associated with polycystic ovary syndrome (PCOS) in patients. The low-fat diet helps to promote weight management and improve insulin sensitivity, two key factors that play a significant role in the metabolic disturbances seen in PCOS. Meanwhile, metformin, a medication commonly used to manage insulin resistance, helps to lower blood glucose levels and restore more regular ovulation cycles (Guan et al., 2020). Together, this dietary approach and pharmacological treatment support the normalization of hormonal imbalances, improve ovarian function, and reduce the risk of long-term complications such as type 2 diabetes and cardiovascular diseases. This combined therapeutic approach can lead to notable improvements in both the metabolic and reproductive aspects of PCOS. (Crave et al., 1995). For patients with polycystic ovary syndrome (PCOS) who are also obese, bariatric surgery has been found to offer significant benefits. This surgical intervention, which involves weight-loss procedures such as gastric bypass or sleeve gastrectomy, helps in achieving substantial and sustained weight loss (Luo et al., 2023). The reduction in body weight can improve insulin sensitivity, normalize menstrual cycles, and reduce the levels of androgens, which are often elevated in women with PCOS. Additionally, bariatric surgery has been shown to alleviate metabolic disturbances associated with PCOS, such as insulin resistance and dyslipidemia, and may help in improving fertility outcomes for those struggling with infertility. While bariatric surgery is not a cure for PCOS, it can significantly improve the overall management of the condition, offering long-term relief from both the metabolic and reproductive complications that often accompany PCOS (Sjöström et al., 2007). In this study a therapeutic approach appears as beneficial for weight management which can bypass the stress and challenges or surgeries.

Laparoscopic Ovarian Drilling (LOD) is a surgical procedure often employed as a treatment for infertility in women with polycystic ovary syndrome (PCOS), particularly when first-line medications like clomifene citrate fail to induce ovulation. This minimally invasive technique involves making small incisions in the ovary to reduce the number of cysts and improve ovarian function, thereby increasing the chances of conception (Mitra et al., 2015). In cases where hormonal imbalances cause irregular menstrual cycles, oral contraceptive pills (OCPs) are commonly prescribed to help regulate these cycles and reduce symptoms such as excessive bleeding and irregularity (Forslund et al., 2023). However, long-term use of OCPs has been associated with certain risks, including an increased likelihood of developing endometrial cancers. Studies have highlighted this potential risk, emphasized the importance of monitoring and evaluated the duration of OCP use, especially in patients who may be at a higher risk for such conditions (Schlesselman, 1997).

Gamma oryzanol, a bioactive compound primarily derived from rice bran oil, typically constitutes around 1-2% of the oil. It is a unique mixture of esters formed from ferulic acid, a phenolic compound, and a variety of sterols and triterpene alcohols. This natural extract has gained attention as a dietary supplement due to its potential health benefits. Research has shown that gamma oryzanol can play a significant role in improving insulin sensitivity, which is crucial for managing conditions such as insulin resistance and type 2 diabetes (Francisqueti-Ferron, Togneri Ferron, et al., 2021). By enhancing the body's ability to respond to insulin, gamma oryzanol may help regulate blood sugar levels and contribute to better metabolic control (Nidhi et al., 2011) mitigating oxidative stress, a process that involves the reduction of harmful free radicals and reactive oxygen species (ROS) in the body, is crucial for maintaining cellular health. Oxidative stress, which occurs when there is an imbalance between the production of free radicals and the body's ability to neutralize them through antioxidants, has been implicated in a wide range of chronic diseases, including cardiovascular disorders, diabetes, and neurodegenerative conditions. By enhancing the body's antioxidant defenses, the reduction of oxidative stress helps protect cells from damage, reduces inflammation, and may slow down the aging process, thereby promoting overall well-being and reducing the risk of disease development. (Francisqueti-Ferron, Garcia, et al., 2021) modulating steroidogenesis, the process by which steroids are synthesized in the body, plays a critical role in maintaining hormonal balance and overall endocrine health. By influencing

key enzymes involved in the production of hormones like estrogen, progesterone, and testosterone, interventions can help regulate steroid hormone levels, potentially correcting imbalances associated with various medical conditions. Additionally, decreasing the xenestrogenic effects those caused by environmental estrogens, or xenoestrogens helps to mitigate the impact of synthetic compounds that mimic estrogen in the body. These compounds, often found in pesticides, plastics, and personal care products, can interfere with the body's natural hormone signaling pathways (Elskens et al., 2023). By reducing exposure to xenoestrogens, one can minimize the risk of hormone-related disorders, such as infertility, certain cancers, and other metabolic issues (Spiazzi CC et al., 2013) in addition to its beneficial effects on insulin sensitivity and oxidative stress, gamma oryzanol has also been shown to have a positive impact on various metabolic parameters. It plays a key role in regulating lipid profiles by helping to lower unhealthy cholesterol levels, including total cholesterol and LDL (low-density lipoprotein), while promoting higher levels of beneficial HDL (high-density lipoprotein) (Seetharamaiah & Chandrasekhara, 1988). Moreover, gamma oryzanol has been found to assist in weight management by supporting the reduction of body fat, potentially through its influence on appetite regulation and fat metabolism. It has also been observed to help lower the Basal Metabolic Rate (BMI), which is an important factor in managing obesity and metabolic syndrome. In addition, gamma oryzanol can contribute to controlling diastolic blood pressure, reducing the risk of hypertension and its associated complications. These combined effects work together to lower overall cardiovascular risk factors, making gamma oryzanol a promising supplement for improving heart health. Furthermore, it has been found to reduce the levels of inflammatory markers, such as high-sensitivity C-reactive protein (hs-CRP), which is often elevated in conditions like chronic inflammation, cardiovascular disease, and metabolic disorders (Kazemzadeh et al., 2014).

Recent research has highlighted the potential benefits of gamma oryzanol in managing lipid levels, particularly in patients suffering from hyperlipidemia. Substantial scientific evidence shows that supplementation with gamma oryzanol can significantly reduce low-density lipoprotein cholesterol (LDL-C) levels, often referred to as "bad" cholesterol, which is a major contributor to the development of atherosclerosis and cardiovascular disease. Additionally, gamma oryzanol has been found to enhance the body's antioxidant capacity, helping to neutralize harmful free radicals and reduce oxidative stress. This dual effect of lowering LDL-C levels while boosting antioxidant

defense makes gamma oryzanol a promising therapeutic option for improving lipid profiles and overall cardiovascular health in individuals with hyperlipidemia (Bumrungpert et al., 2018).

Gamma oryzanol, available in various commercial formulations such as HI-Z, is commonly used in the management of hyperlipidemia and inflammatory bowel disease (IBD), where it has shown potential benefits in improving lipid profiles and reducing inflammation. This supplement, which is derived from rice bran oil, has gained attention for its therapeutic effects in these conditions, particularly in regulating cholesterol levels and supporting gut health. However, despite its known advantages in these areas, the effects of gamma oryzanol on polycystic ovary syndrome (PCOS) have not yet been thoroughly investigated. As of now, there is a lack of clinical research or studies evaluating how gamma oryzanol might influence the hormonal and metabolic disturbances associated with PCOS, leaving a gap in understanding its potential role in managing this complex endocrine disorder (HI-Z Tablets 25mg | Kusuri-No-Shiori(Drug Information Sheet), 2024).

Morinda citrifolia known as Noni, is widely found in the Asia (mostly south-east part), Australia, New Zealand. This *rubiaceae* fruity plant also known as “Indian mulberry”. Noni plant is traditionally used in various countries like Polynesia, Hawaii as a general health tonic. The fruit is also used as a staple food in many regions.

The medicinal value of noni plant is established by various studies. *Morinda citrifolia* contains lignan which regulates testosterone levels in male rabbits while fed 200,400 and 800mg/kg noni juice. The serum testosterone level was found to be decreased (Sukardi et al., 2005).

Insulin sensitivity is also found to be modulated with noni fruit extract at 250 and 500 mg/kg to Swiss mice. The 500mg/kg dose exhibits higher glucose tolerance (Inada et al., 2020).

Anticancer activity is established with noni fruit extract in lung cancer and breast cancer (Sharma et al., 2015) (Hirazumi et al., 1994).

Oxidative stress is also reduced while treated with noni. The HeLa and SiHa cervical cancer cell lines was treated with noni juice (10%). Lipid peroxidation was decreased, and CAT (catalase) activity was increased on this study (Gupta & Singh, 2013b).

Noni also exhibits antibacterial, antiviral, anthelmintic, analgesic, hypotensive, immunological activity and improvement of mental health, increasing hearing ability in various studies (Mian-Ying et al., 2002).

With a focus on these specific health parameters, we have conducted an evaluation to assess the potential ameliorative effects of gamma oryzanol, in combination with *Morinda citrifolia*, in the management of polycystic ovary syndrome (PCOS). This study aims to explore how these two natural compounds, individually and synergistically, might influence the hormonal imbalances, metabolic disturbances, and other clinical manifestations characteristic of PCOS, offering a novel approach to managing this complex condition.

PCOS patients exhibit elevation of the levels of certain steroid hormones, such as estrogen and testosterone, are typically elevated. These hormonal imbalances are a hallmark of PCOS and contribute to many of the condition's characteristic symptoms, including irregular menstrual cycles, ovulatory dysfunction, and signs of hyperandrogenism, such as acne and hirsutism. The dysregulation of steroidogenesis in PCOS leads to an excess of androgens (testosterone), which further disrupts the balance of estrogen and other key hormones. (Kokabiyan et al., 2022). This research, treatment with gamma oryzanol and *Morinda citrifolia* resulted in a significant decrease in the levels of estrogen, suggesting that these treatments may help restore hormonal balance in the context of polycystic ovary syndrome (PCOS). A similar reduction was observed in testosterone levels, indicating that both compounds may have a beneficial effect in lowering the elevated androgen levels typically associated with PCOS. These findings suggest that gamma oryzanol and *Morinda citrifolia* could play a role in modulating steroid hormone levels and potentially alleviating some of the hormonal disturbances seen in PCOS.

In this study, we induced polycystic ovary syndrome (PCOS) in an animal model by administering estradiol valerate, a synthetic estrogen compound known to mimic the hormonal imbalances seen in women with PCOS. This method was chosen to replicate the key features of the condition, allowing for a controlled investigation into the effects of various treatments on the pathophysiology of PCOS (Kokabiyan et al. 2022) (Amini et al. 2016). After a period of 28 days, PCOS was successfully induced in the animal model. Following this, we administered gamma oryzanol and Morinda citrifolia at two different dosage levels, along with the standard treatment

of clomifene citrate. We then closely observed the individual and combined effects of these treatments on various clinical and biochemical parameters associated with PCOS. This approach allowed us to evaluate the potential synergistic effects of gamma oryzanol, Morinda citrifolia, and clomifene citrate when used together, as well as their individual contributions to improving the key aspects of PCOS.

In the current study, treatment with gamma oryzanol and *Morinda citrifolia* resulted in a notable increase in the activity of critical antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). These enzymes are vital to the body's defense mechanisms against oxidative stress, as they work to neutralize damaging free radicals and reactive oxygen species. The observed enhancement in the activity of these antioxidants suggests that gamma oryzanol and *Morinda citrifolia* may help mitigate oxidative damage, thereby reducing oxidative stress in the system. The observed increase in antioxidant activity suggests a potential therapeutic benefit of these compounds in managing conditions like polycystic ovary syndrome (PCOS), where oxidative stress is a key factor in the progression of the disease. This enhancement in antioxidant defense may help mitigate the damaging effects of oxidative stress and support overall health in individuals with PCOS.

Obesity is a prominent clinical feature commonly allied with polycystic ovary syndrome (PCOS), contributing to various metabolic and hormonal disturbances. In the contemporary study, we perceived a considerable rise in body weight in the PCOS-induced groups, reflecting the typical weight gain seen in this condition. However, in the groups treated with gamma oryzanol and *Morinda citrifolia*, a noticeable reduction in body weight was recorded. This suggests that both gamma oryzanol and *Morinda citrifolia* may have a beneficial effect in mitigating weight gain and possibly improving metabolic outcomes in PCOS patients. The weight-reducing effect of these compounds could be linked to their ability to regulate hormonal imbalances and enhance metabolic function, offering a potential therapeutic approach for managing obesity in PCOS.

In the group treated with gamma oryzanol and *Morinda citrifolia*, a significant decrease in ovarian weight was observed, which was consistent with the reduction in the number of follicles. This change was further corroborated by histopathological examination, which revealed a notable decline in follicular count. The reduction in both ovarian weight and follicle number suggests that

these treatments may help regulate ovarian function, potentially reversing some of the pathological changes typically seen in polycystic ovary syndrome (PCOS). This histological finding underscores the potential therapeutic effects of gamma oryzanol and *Morinda citrifolia* in modulating ovarian health and improving the overall reproductive function in PCOS.

The individuals diagnosed with polycystic ovary syndrome (PCOS), a disruption in estrous cyclicity is commonly observed, where the normal progression and synchronization of the four distinct phases of the cycle are impaired. This imbalance leads to irregular or absent cycles, which is a hallmark of the condition. In the present study, we demonstrate that treatment with gamma oryzanol and *Morinda citrifolia* can effectively reverse this disruption, restoring the proper sequence and regularity of the estrous cycle. This suggests that both compounds may help restore normal ovarian function and hormonal balance in PCOS.

Infertility is one of the primary clinical outcomes associated with PCOS, often linked to hormonal imbalances and anovulation. To assess the fertility potential in the PCOS-induced animals, we evaluated the number of implantation sites, which serves as an indicator of successful embryo implantation and early pregnancy. Our findings indicate that treatment with both gamma oryzanol and *Morinda citrifolia* significantly increased the number of implantation sites in PCOS animals, suggesting that these treatments may enhance fertility and improve reproductive outcomes in PCOS.

Polycystic Ovary Syndrome (PCOS) is a diverse disorder characterized by menstrual irregularities, the presence of multiple ovarian cysts, androgen and testosterone levels elevation, too obesity along with insulin resistance kind of metabolic disorders are the common syndromes of PCOS. All those pathological features collectively define the essence of PCOS. The existing management approaches have shown limited effectiveness, providing only symptomatic relief. Therefore, it is imperative to explore novel strategies to address this complex disorder.

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Summary

Nutraceuticals are considered as a prime target for PCOS management in recent studies.

Gamma oryzanol derived from rice bran and Morinda citrifolia known as Noni plant has been traditionally used as a dietary component in various cultures across the globe.

Rice bran oil which is rich in gamma oryzanol is commercially available which is globally accepted as a healthier alternative of traditional edible oils.

Gamma oryzanol is also available as a prescribed medicine which helps to lowering the cholesterol level and as a modulator of endocrine system. Gamma oryzanol in the brand name of Hi-Z manufactured by Otsuka Pharmaceutical Co. Ltd. is available with 25mg tablet form which can be taken 100mg/day for an adult to eradicate hyperlipidemia.

In this study we observed that, gamma oryzanol brings back the regularity in estrous cycle which was disrupted in the PCOS condition. The histology also shows a significant change in ovarian cellular morphology which demonstrate the regeneration of folliculogenesis. The changes in hormonal levels are found to be reversed by decreasing the level of estrogen and decreasing the level of progesterone which in turn helps folliculogenesis, improve estrous cyclicity. The oxidative stress is also observed to be reduced with gamma oryzanol which was achieved by maintaining level of catalase, SOD and GSH. Increase body weight is known as a clinical feature of PCOS which is also reversed in our study by the use of gamma oryzanol. The ovarian weight found to be reduced in the rats who are treated with gamma oryzanol the possible reason behind this is the less cystic structure present in ovary which is also proven by the histological study. In PCOS the clinical outcome is infertility. Here gamma oryzanol showed a fertility improvement potential in PCOS conditions.

Morinda citrifolia is rich in “lignin” content. “Lignan” is a phytoestrogen which act like an estrogen in body. *Morinda citrifolia* also known as “Noni” is used as anti-aging therapy, diabetes, high blood pressure, in sports as a performance increasing supplement. The leaves, fruits, stem, bark even flowers all are used as a medicinal component.

In this study we observed that, Noni maintains the regularity in estrous cycle in the PCOS condition. The histology with Noni we observed that there is a significant change in ovarian

cellular morphology which demonstrate the regeneration of folliculogenesis. The changes in hormonal levels are found to be reversed by decreasing the level of estrogen and decreasing the level of progesterone which in turn helps folliculogenesis, improve estrous cyclicity. The oxidative stress is also observed to be reduced with Noni that was achieved by maintaining level of catalase, SOD and GSH. Increase body weight was also reversed in our study by the use of Noni. The ovarian weight found to be reduced in the rats who are treated with Noni, the possible reason behind this is the less cystic structure present in ovary which is also proven by the histological study. The fertility score is also improved with the use of Noni in PCOS.

In this study we tried combination of test drugs with themselves and also with standard drug individually and in a combination of all three. We used clomifene citrate as a test drug which is a renowned drug used to treat patient suffering with PCOS.

In this study the combinations were created with 1. gamma oryzanol with clomifene citrate, 2. Morinda citrifolia with clomifene citrate, 3. gamma oryzanol with Morinda citrifolia and finally 4. gamma oryzanol, Morinda citrifolia with clomifene citrate.

Gamma oryzanol with clomifene citrate shows a synergistic effect in comparison with the gamma oryzanol only treated group and shows additive effect in comparison with clomifene citrate only treated group.

Morinda citrifolia with clomifene citrate shows a synergistic effect in comparison with the Morinda citrifolia only treated group and shows additive effect in comparison with clomifene citrate only treated group.

The combination of two test drugs gamma oryzanol with Morinda citrifolia shows a synergistic effect in comparison with the individual treatment groups.

Combining gamma-oryzanol and *Morinda citrifolia* with clomifene citrate produces an additive effect, surpassing the effects observed when combined with either the standard or test drug alone.

Conclusion

From this study we can conclude that, gamma oryzanol and *Morinda citrifolia* exhibit a potential candidate for the treatment of polycystic ovarian syndrome. They both have an effect on regulating

menstrual cycle. In the ovarian steroidogenesis point of view, they both can control the levels of estrogen and testosterone. As a result, they both is capable of improving folliculogenesis. In PCOS oxidative stress always on higher side, our test drugs gamma oryzanol and *Morinda citrifolia* is observed to reduce the oxidative stress. The body weight is maintained with the help of these two components. The ovarian morphology shows the disruption caused by PCOS is improved with the use of these two test drugs. The decrease of ovarian weight with the gamma oryzanol and *Morinda citrifolia* is the observation of reversal of cysts. All this benefits finally summarized in the increase of fertility rates in the rats.

In this study we not only aimed for betterment of fertility but also targeted the quality of life of a woman's reproductive age. PCOS causes a significant mental challenge along with hampering day to day lifestyle due to disruption of menstrual regularities. The various parameters we evaluated—except for the fertility study, such as steroidogenesis and menstrual cyclicity—showed positive results. These findings could be beneficial for individuals at different stages of their reproductive age, not only for those who are specifically aiming for conception. Hence, we can say that gamma oryzanol and *Morinda citrifolia* can be used as an ameliorative treatment approach in the PCOS condition.

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Gamma Oryzanol: A natural compound with potential for treating polycystic ovary syndrome

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ABSTRACT

Introduction: Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age. Current management strategies only provide symptomatic relief, and there is a need for better treatments. Gamma-oryzanol, a dietary component found in rice bran oil, has been shown to be effective in increasing insulin sensitivity, reducing weight, and alleviating oxidative stress. In modern Chinese medicine, 香格里拉谷维素胶囊, 纯净谷维素软胶囊, and 诺特博谷维素片 are some of the medicines marketed in China that contain gamma-oryzanol. This study aims to evaluate the effects of gamma-oryzanol on clinical hallmarks of PCOS. Also to compare the efficacy of gamma-oryzanol alone versus in combination with clomifene citrate.
Methods: In this study, PCOS was induced in a rat model using estradiol valerate. Gamma-oryzanol was then administered both alone and in combination with clomifene citrate.
Results: The effects of gamma-oryzanol on estrous cyclicity, fertility, histopathological alterations, estradiol and testosterone levels, and oxidative parameters were evaluated. The results showed that gamma-oryzanol, both alone and in combination with clomifene citrate, had protective effects against the estradiol valerate-induced PCOS rat model. The combination of gamma-oryzanol and clomifene citrate was particularly effective, showing significantly better results than the standard treatment group (clomifene citrate alone).
Discussion: These results suggest that gamma-oryzanol could be used in combination with existing standard treatments to enhance the management of PCOS. However, further molecular and clinical studies are needed to confirm these findings.

Introduction

PCOS is considered the most common endocrinological disorder among women of reproductive age, with a prevalence of 4 to 4.7 % in United States [1]. In Europe, some studies have reported that the prevalence of PCOS is between 420 and 440 cases per 100,000 women [2,3].

PCOS is a metabolic syndrome and complete cure from PCOS is yet not available. PCOS management can be achieved through lifestyle modifications and medications, including diet, exercise, and pharmacological treatments. (ex: Clomifene Citrate, metformin (Glucophage) (Glucophage), Oral contraceptive pills, spironolactone (Aldactone) etc) [4–7] and Dietary supplements (ex: Chromium, Selenium, Omega 3 fish oils, Vitamin D, Inositol etc.) [8–12].

Animal models are essential tools for studying the underlying mechanisms of diseases. Researchers have conducted various animal studies to better understand the pathophysiology and etiology of PCOS. This complex disorder is characterized by insulin resistance, elevated androgen levels, infertility, and menstrual irregul arities. These metabolic complications can lead to obesity and other features of metabolic syndrome. Animal models have provided valuable insights into the roles of insulin resistance, androgens, and other factors in the development of PCOS. Additionally, these studies have helped identify potential targets for new treatments [13].

Numerous animal studies have shown that PCOS-like symptoms can be induced by interventions involving testosterone, estrogen, and androgens, as well as by environmental disruptors [14–25]. DHEA, letrozole (Femara), d-galactose, Bisphenol A, Monosodium-l-glutamate are also found to be causative chemical compound of PCOS [26–29].

PCOS does not have any direct treatment approach. Symptomatic relief and improvement of fertility are considered as PCOS management strategies. However, various current researches tried to combat with

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This is to certify that Prof./Dr./Mr./Ms. Sayantika Chakraborty has successfully participated as Delegate & Presented Poster/ Oral Presentation on Ameliorative effect of Gamma Oryzanol and Mosinola Citrifolia in PCOS using animal models in the 3rd International Conference of Pharmacy (ICP-2022) on the Theme of "Practice, Promotion & Publication of Innovation : A Way of Transforming Health" held on 09th & 10th November 2022 organized by School of Pharmaceutical Sciences in a collaboration with Indian Pharmaceutical Association (IPA) at Lovely Professional University, Punjab.

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