



Evaluation of the Therapeutic Effects of *Tribulus Terrestris* Extract on Letrozole-Induced Polycystic Ovarian Syndrome (PCOS) in Female Wistar Rats

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Abstract

Polycystic Ovarian syndrome (PCOS) is a intricate endocrine disorder which is described by hyperandrogenism, anovulation, and metabolic disturbances, often modeled experimentally using letrozole-induced ovarian dysfunction in rodents. The present study evaluated the beneficial potential of *T. terrestris* extract in comparison with metformin in female rats with Wistar strain with letrozole-induced PCOS. 30 rats were divided into five groups: normal control, letrozole control, low-dose *T. terrestris* (100 mg/kg), high-dose *T. terrestris* (200 mg/kg), and Metformin (300 mg/kg). Parameters assessed included food consumption, body weight, estrous cycle, hematology, serum hormone concentrations, organ weights, and ovarian histopathology. Letrozole disrupted increased body weight, Estrous cyclicity altered hematological indices, and induced ovarian cysts. Treatment with *T. terrestris* extract restored estrous cyclicity, normalized serum gonadotropins and steroid hormones, improved hematological indices, and ameliorated ovarian histopathology, with effects comparable to metformin. The higher *T. terrestris* dose showed slightly stronger efficacy. These findings suggest that *T. terrestris* exerts estrogenic and anti-androgenic actions capable of counteracting PCOS-associated metabolic and reproductive disturbances.

Keywords: Polycystic Ovarian syndrome (PCOS); Letrozole-induced model; *Tribulus terrestris*; Metformin; Estrous cycle.

Introduction

Polycystic Ovarian syndrome is the most major endocrine and metabolic syndromes, affecting women of reproductive age with a global popularity ranging between approximately 6% to 20% depending on the diagnostic criteria applied [18]. Stein-Leventhal Syndrome, the previous name for PCOS, was first identified in 1935 by American gynecologists, Irving F. Stein Sr. and Michael L. Leventhal [59]. It is distinguished by a complex pathophysiology involving amenorrhea oligomenorrhea and hyperandrogenism, and polycystic ovarian morphology. PCOS is often linked with chronic metabolic syndromes such as insulin resistance, obesity, dyslipidemia, endometrial cancer, cardiovascular diseases and a high risk of having type 2 diabetes [61]. The etiology of the PCOS is still obscure. One hypothesis is that PCOS is an inherited ovarian disease in which excess androgens in early life led to PCOS in adulthood [40]. The World Health Organization (WHO) stated that PCOS has affected over 116 million of women worldwide in 2012[67]. One in five Indian women is affected by PCOS [67]. A large-scale survey conducted across India in 2020 showed that around 16% of female respondents between the ages of 20 to 29 years suffered from PCOS [1].

The in vivo and laboratory studies advocated that ovarian theca interna cells have increased activity of converting androgenic predecessors into testosterone in the women having polycystic ovarian syndrome than in a healthy woman. Certainly, the ovarian theca interna cells produce androgen in retortion to the luteinizing hormone (LH). Thus, the blood level of androgen are elevated in PCOS [53]. The elevated levels of androgens particularly testosterone in polycystic ovarian syndrome, results to decrease in ovulation and interrupt the formation of sex steroids (Gonadal steroids), which effects clinical symptoms such as hirsutism, alopecia, seborrhea and reproductive tract dysfunction in the sufferers, are the main causes for infertility in a reproductive age women [37],[8]. The reason of infertility is an absence of ovulation in nearly 75% of the cases [62].

As per the Rotterdam, PCOS is distinct when there are presence of two of three of the subsequent criteria: oligo-anovulation, hyperandrogenism and polycystic ovaries (≥ 12 follicles measuring 2- 9 mm in diameter or an ovarian volume > 10 mL in at least one ovary) [54].

Compensatory hyperinsulinemia and Insulin resistance further impair the hyperandrogenic state by stimulating androgen synthesis and inhibiting hepatic sex hormone-binding globulin (SHBG) production, increasing free testosterone and estrogen levels [17]. This dual action of insulin enhances the androgenic conditions and

contributes to the metabolic disturbance observed in polycystic ovarian syndrome patients [21]. Currently, Tamoxifen, metformin, and Clomiphene citrate are the drugs to use to treat PCOS [42]. But these drugs can cause the severe effects of such medicines and the relative treatment of PCOS by them. It is important to identify and discover alternative drugs [32]. which plant-based drugs, particularly estrogen having ones (Phytoestrogens) are contemplated to be relatively more effective [7]. Phytoestrogens are less antagonists of estrogen and they display more effective estrogenic effects when the level of estrogen in the body is decreased [63]. *Tribulus terrestris* L. (a affiliate of the Zygophyllaceae family) is commonly known as Tribulus, Hard thorns and goat head in China, is a perennial plant widespread in the Mediterranean area, Subtropical and deserts around the world [3],[11]. It is widespread as a sexual activity enhancer, and it is broadly in traditional herbal medicine. Investigations into its pharmacological actions illustrate that *T.terrestris* could improve the system of reproduction in both genders. It could upsurge the concentration of hormones such as Estradiol and very slightly testosterone which could enhance male and female libido, oogenesis, spermatogenesis, and ovulation [2],[22],[41],[20],[34],[27]. Several studies have stated the fundamental role of secondary metabolites from *T.terrestris* [15],[73]. The presence of steroidal saponins could be responsible for natural hormonal efficiency by directly stimulating female endocrine-sensitive tissues such as the vagina and uterus [47]. Many studies confirmed the fundamental role of secondary metabolites of *T.terrestris* and further considered each specific medicinal property of the plant to its secondary metabolites. In this respect, the main secondary metabolites of *T.terrestris* include steroidal saponins, glycosides, phytosterols, flavonoids tannins, terpenoids, amide derivatives, amino acids, and proteins [15],[73]. The positive effects of *T.terrestris* on the reproductive system of female were termed to be primarily caused by the presence of Protodioscin (a steroidal saponin compound) in this plant [26]. The phytoestrogens present in *T.terrestris* bind with estrogen receptors ER α and ER β and acts as a pure estrogen antagonist by stimulating gonadotropin-releasing hormone secretions. Such a mechanism normalizes the hormonal level, induces ovulation and reduces the ovarian cysts in PCOS [48].

The hydroalcoholic extract of *T.terrestris* normalizes the menstrual irregularity and hormonal alterations, efficiently removes the Ovarian Cysts and resumes ovarian normal activity in the Estradiol valerate-induced PCOS rats. It might be due to its luteinizing effect related to gonadotropin-like activity or luteinization of follicular cysts [13],[19]. A study revealed that *T.terrestris* has an LH-like activity that induces corpus luteum formation and thus starts puberty. Moreover, the observed enhancement in corpus luteum diameter may relate to the increase in progesterone production [56]. *Tribulus terrestris* has the potential to modulate ovarian physiology by promoting luteinization of the follicular cysts, potentially through endocrine or paracrine mechanisms. Experimental studies have stated a substantial growth in the number of animals exhibiting corpus luteum formation, suggesting the re-establishment of normal estrous cycles [13]. Another study indicated the presence of phytoestrogens in *Tribulus terrestris*, which may affect the brain's regulation of sex hormone secretion and enhance its antioxidant potential [56]. Furthermore, *T. terrestris* has been reported to exert regulatory effects on the estrous cycle of PCOS-induced rats by reducing steroid hormone levels. Reports have showed that extracts of *T.terrestris* significantly decreased the dihydrotestosterone suggesting an anti-androgenic property of the plant. Additionally, extract-treated rats exhibited a significant improvement in body weight gain and a marked reduction in serum testosterone concentrations [57].

Protodioscin known as Saponin according IUPAC is 26-O- β -D-Glycopyranosyl-22-hydroxyfurost-5-ene-3 β ,26-diol-3-O- β -diglucorhamnoside. Besides *Tribulus*, it has been identified in a number of plant species such as *Trigonella* and *Dioscorea* families [24],[31],[16]. Thus, Protodioscin has been isolated as ethanol extract from rhizomes of *Dioscorea panthaica*

L. [31] and purified and identified from *Trigonella foenumgraecum* L. [16].

Steroidal saponins are normally occurring glycosides that have properties such as producing foam, complex formation with cholesterol. Steroidal saponins are found as plant secondary metabolites or a compound that helps proteins go through cellular membranes [45]. Steroidal saponins are extracted from the *Tribulus terrestris* plant. *Tribulus terrestris* contains a number of nutritional supplements along with 45% and 95% steroidal saponins. Protodioscin is the most important among them. Protodioscin is assumed to raise androgen receptor concentrations in cellular structures, encouraging those structures to become more susceptible to androgen like testosterone [14],[60]. Letrozole is an aromatase inhibitor that averts the level of testosterone from converting to estradiol, thereby increasing blood and ovarian androgen levels [10],[52]. Aromatase is a small member in the family of the cytochrome P450 which converts androstenedione to estrogen and then testosterone to estradiol [52]. Letrozole induces similar PCOD changes in comparison with PCOS women such as increased weight and size of ovary, thickens the theca interna cell layer, anovulation, and hyperandrogenism [40],[6],[74].

Metformin, a biguanide class oral antidiabetic agent, is used in the preliminary line pharmacological therapy in women suffering polycystic ovarian syndrome (PCOS) who are having insulin resistivity. The primary working of metformin involves the obstruction of hepatic gluconeogenesis through initiation of AMP-activated protein kinase (AMPK), a that has cellular energy sensors which in turn regulates glucose and lipid metabolism. Initiation of AMP-activated protein by metformin decreases hepatic glucose production, increases peripheral glucose uptake, and enhances insulin sensitivity in adipose tissue and skeletal muscle [51]. Women suffering with PCOS, this action helps mitigate hyperinsulinemia, thereby reducing insulin-stimulated production of androgen in ovary and improving menstrual cyclicality [44]. Moreover, metformin has been showed to decrease luteinizing hormone (LH) levels and improve follicular maturation, supporting its role in restoring reproductive function [46]. In addition to its metabolic effects, metformin exerts anti-inflammatory and antioxidant

properties, which may further contribute to its therapeutic benefit in PCOS by modulating systemic inflammation and oxidative stress, both of which have known to show the pathophysiology of the syndrome [29].

Materials and methods Ethical approval

The animal experiment had received its approval from the Institutional Animal Ethics Committee (IAEC) as identified by approval number (SUK/ZOL/IAEC/05/2024-25) through Sharnbasva University Department of Postgraduate Studies and Research in Zoology in Kalaburagi, Karnataka, India. (CCSEA Regd. No: 2236/PO/ReBiBt/S/23/CCSEA).

Experimental Animals

The 30-adult female with strain of Wistar albino rats (*Rattus norvegicus*), 6–8 weeks old, weighing about 120–150 g, were bred in the animal house facility, Department of PG Studies and Research in Zoology, Sharnbasva University, Kalaburagi. (SUK/ZOL/IAEC/05/2024-25). All the animals were kept in well-ventilated and temperature-controlled rooms. Water and a standard diet were provided ad libitum and monitoring of body weights were done weekly.

Experimental Setup

Rats were housed in an optimum condition with room temperature: $21\pm 3^{\circ}\text{C}$ with relative humidity between 58 and 67 %; 12 hours of light and 12 hours of dark cycle. The sterilized paper shreds were provided as nesting material. Rats were placed in the polyethylene cages with paddy husk. Pellet rodent feed (Chompaka Feeds and Foods, Bangalore, India) and purified water in polycarbonate bottles were provided ad libitum.

Experimental design

The division of 30 wistar strain of female rats were divided into 5 groups, 6 rats in each group. 2 cages per group. The experiment was divided into 2 phases, Phase 1 - PCOS induction; Phase 2 – treatment phase. Administration and duration of dosing of letrozole for induction of PCOS was in accordance to previous studies.

Groups and Dosing

Administration method: oral gavage

I. Phase 1 – PCOS induction phase [day 1-21] Duration 21 days

For 21 days the rats received Letrozole 1 mg/kg/day to induce PCOS

- a. Group 1: Normal Control - Rats received 1% CMC orally as vehicle.
- b. Group 2-5 – Rats were given Letrozole 1 mg/kg/day orally via gavage throughout Phase 1 for 21 days for induction of PCOS.

II. Phase 2 – treatment phase [day 22-62] Duration: 40 days

After the completion of 21 days, 2 rats from group 2 were euthanized and the ovaries were removed and observed for formation of cyst and Irregularity of Estrus Cycle, i.e., recurring Diestrus Phase confirming PCOS.

1. Group 1: Normal control
2. Group 2: PCOS control – non-treated
3. Group 3: Low dose – Rats were received *T. terrestris* extract by oral gavage at low dose 100 mg/kg/day for 40 days.
4. Group 4: High dose – Rats were received *T. terrestris* extract by oral gavage at high dose 200 mg/kg/day for 40 days.
5. Group 5: Standard drug Metformin – Rats were received Metformin by oral gavage 300 mg/kg/day for 40 days.

Chemical preparation

1. 1% CMC (Carboxymethyl Cellulose)

It was prepared by dissolving 1g of Carboxymethyl cellulose (CMC) powder in 100 ml of distilled water gives 1% CMC.

2. Letrozole

It was prepared by crushing the tablets of 2.5 mg/tablet and dissolving the powder in 1% CMC.

0.2 mg of letrozole powder was dissolved in 4 ml of 1% CMC for a rat weighing 200 g. A stock solution of 1 mg/kg for 7 days was prepared according to the animals' body weight.

3. *Tribulus terrestris*

The solution was prepared by dissolving the powdered extract in distilled water.

1. Low dose: It was prepared by dissolving 20 mg powdered extract in 4 ml distilled water to obtain the dose of 100 mg/kg for a rat weighing 200 g. Stock solution for 7 days was prepared according to rats body weight.

2. High dose group: 40 mg powdered extract was dissolved in 4 ml distilled water to obtain the dose of 200 mg/kg for a rat weighing 200 g. Stock for 7 days was prepared according to the rats body weight.

Metformin

It was prepared by crushing tablets of 500 mg/tablet and dissolving them in 1% CMC. As per the experimental design, the animals of group 5 received a dose of 300 mg/kg/day. 60 mg of metformin powder was dissolved in

2 ml of 1% CMC to obtain a 300 mg/kg dose for a rat weighing 200 g. The stock for 7 days was prepared according to the animals' body weight.

Estrous cycle monitoring

The estrus cycle was monitored at 9:00 am for 61 days by taking vaginal smears and observing them under a microscope after staining. The observation was made for a consistent diestrus phase to confirm the induction of PCOS during the phase-1(induction of PCOS) of the experiment. Then, the observation was made for the estrus cycle restoration of the treatment group during the Phase 2 (treatment phase) of the experiment.

Body weight and Food consumption

The rats were chosen randomly, of 6-8 weeks of body weight, weighing around 120-150 g. The body weights were measured weekly. Each cage consists of 3 animals provided with 400 g of food weekly and a record is maintained of food consumed and food remaining in each cage weekly. The lethal body weight was assessed before the animal sacrifice. The animals underwent a period of fasting before necropsy.

Blood and tissue collection

1. On day 21, two rats from Group 2 - PCOS control were euthanized and necropsy was performed, and the obtained ovaries observation was done under microscope.
2. On day 63, rats from all the 5 groups were weighed and euthanized. Blood sample was collected in blood vials with ice-cold packet. The ovary, uterus and other vital organs were excised, trimmed of adherent fat, washed in normal saline water and weighed. All reproductive and vital organs were fixed in 10% formalin solution for histopathological study.

Hematology

The blood for Hematological examination was collected on day 63. All the rats were deprived of food throughout the whole night before blood extraction. The collection of blood was done through cardiac puncture. The parameters of hematology were assessed such as Hemoglobin, RBC's, WBC's - Neutrophils, Lymphocytes, Eosinophils, Monocytes., Platelet count, Mean Corpuscular Hemoglobin Concentration (MCHC) Hematocrit (PCV), Mean Corpuscular Hemoglobin (MCH) Red Cell Distribution Width (RDW), Mean platelet volume (MPV) Mean Corpuscular Volume (MCV).

i.Hormonal assay

Blood was collected by cardiac puncture between 9:30 AM and 11:30 AM for hormonal analysis. The measured parameters included serum concentrations of FSH (mIU/mL), LH (mIU/mL), Estradiol (pg/mL), Progesterone (ng/mL) which were quantified using the Electro-Chemiluminescence Immunoassay (ECLIA) method.

Histopathological analysis

Euthanasia was performed on 63-day-old rats using Isoflurane, followed by surgery to extract their organs. The extracted organs were then placed in containers filled with 10 % formalin, and Haematoxylin and Eosin (H & E) staining were used during Histopathological preparations.

Statistical analysis

All data were articulated as Mean $\pm$ Standard Deviation (SD) and Standard Error (SE). Accomplished Statistical analysis was done using GraphPad Prism 10 software, utilizing two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to analyse differences between groups.

Results Clinical signs

Throughout the duration of the study, there was no mortality in the divided experimental groups. All animals appeared healthy and exhibited normal behaviour. No treatment-related clinical signs of toxicity such as piloerection, salivation, lethargy, changes in locomotor activity, or abnormal posture were recorded in any group during either the induction or treatment phases.

Food consumption

At the letrozole induction phase, a progressive increase in food intake was initially observed in all groups between Day 7 and Day 14, with Group 5 demonstrating the highest Mean intake on Day 14 (17.52 ± 0.57 g/day). No substantial differences were observed in all groups during this phase. Over the 40-day treatment period, food consumption showed significant variation among the experimental groups. Animals treated with Letrozole demonstrated moderately reduced food intake compared to the normal control. Administration of Tribulus terrestris extract at both 100 mg/kg and 200 mg/kg produced dose-dependent increases in Mean food consumption, with statistically noteworthy improvements observed from Day 28 onwards ($p < 0.05$ or $*p < 0.01$ vs. Letrozole group). The standard drug Metformin consistently elevated food intake, particularly at Day 56 and Day 62 ($*p < 0.01$), indicating its potential efficacy in ameliorating Letrozole-induced reductions in feeding behaviors.

Body weight

Body weight changes in the letrozole-induced PCOS model showed clear patterns across the two experimental phases. During the 21-day induction period, all letrozole-treated groups (Groups 2–5) gained apparently more

weight than the Normal Control group (Group 1; $p < 0.05$). The Metformin group (207.0 ± 11.4 g) and the high-dose *Tribulus terrestris* group (210.7 ± 5.5 g) showed a 23–25% higher weight compared to controls (168.3 ± 6.3 g), confirming letrozole's weight-promoting effect.

After starting treatments (Days 22–65), differences became clearer. The Letrozole Only group (Group 2) showed minimal further gain, reaching 185.0 ± 90.7 g by Day 65. In contrast, all treatment groups (Groups 3–5) continued to gain weight, reaching 231–235 g ($p < 0.05$ vs. Group 2). Notably, the high-dose *T. terrestris* (200 mg/kg, Group 4) and Metformin (Group 5) groups had similar final weights (around 235 g), showing comparable treatment effectiveness. Interestingly, the normal control group also showed some late weight gain (204.8 ± 6.1 g), but this remained 15% lower than the treated groups.

Statistical analysis showed a significant interaction between group and time ($F(40,1200) = 5.2$, $p < 0.001$), with treatment effects becoming evident from Day 22 onward ($p < 0.05$). No clear dose-dependent effect was seen between 100 and 200 mg/kg of *T. terrestris* ($p = 0.78$). Overall, both *T. terrestris* and Metformin helped maintain the increased body weight caused by letrozole, indicating similar metabolic benefits.

Estrous cycle

LETROZOLE INDUCTION PHASE (DAYS 1–21)

Induction with letrozole for 21 days produced pronounced changes in the estrous cycle (Table 5.4.1, graph 5.4.1). Compared with controls, letrozole-treated groups spent least time in estrus and metestrus and most time in diestrus. The estrus Mean fell from

~4.2 days in controls to 0.6–1.6 days in the letrozole groups ($p < 0.01$), while diestrus increased from ~11 days to >16 days ($p < 0.01$). Proestrus counts were modestly reduced; only group 5 showed a significant decrease. These findings indicate that letrozole induces a persistent luteal-like state (diestrus) with minimal follicular activity.

T. TERRESTRIS TREATMENT PHASE (DAYS 22–62)

After induction, the untreated letrozole group (Group 2) remained acyclic during the final 21 days, spending the entire period in diestrus with no estrus or metestrus episodes (Table 5.4.2, Plate 5.4.1). Treatment with *T. terrestris* or metformin restored cyclicity. The 100 mg kg⁻¹ dose (Group 3) produced estrus/metestrus counts similar to the control and no significant differences were detected. The 200 mg kg⁻¹ dose (Group 4) further increased estrus days (7.2 ± 1.1 days vs 5.4 ± 1.1 in controls; $p < 0.05$) and reduced diestrus days. Metformin (Group 5) also tended to increase estrus days but they did not differ considerably from controls. Proestrus counts in the letrozole and herbal/Met groups were comparable to controls except for the untreated letrozole group (zero days; $p < 0.01$).

ORGAN WEIGHT

Organ weight analysis across five groups exhibited no statistically substantial differences for most of the organs when compared to the normal control (Welch's *t*-test, $p > 0.05$). Significant differences ($p < 0.05$) were observed in: Brain – Group 3: *T. Terrestris* 100 mg/kg/day vs Control ($p = 0.0081$).

Hematology

Hematological analysis revealed distinct treatment-related effects. Total WBC count showed significant alterations across groups, with the Letrozole group exhibiting elevated leukocyte levels compared to controls, while both *Tribulus terrestris* and Metformin-treated groups demonstrated a partial normalization of values. Lymphocyte percentage was markedly reduced in the treatment groups relative to the control, with significant differences confirming immune-modulatory effects. Similarly, monocyte counts were significantly altered, indicating possible inflammatory responses. Hematocrit values were substantially elevated in the Letrozole group but tended to normalize following treatment with *Tribulus terrestris* and Metformin, reflecting a restorative effect. In contrast, parameters such as neutrophils, eosinophils, RBCs, hemoglobin, MCV, MCH, MCHC, platelets, and MPV did not show statistically considerable differences, suggesting these indices were relatively unaffected by the treatments.

Hormonal assay

The hormonal results indicated substantial differences in all the five groups for all hormones (FSH, LH, estradiol, progesterone; all $p < 0.001$). Versus the normal control, letrozole decreased FSH, estradiol, and progesterone, and increased LH (Tukey $p < 0.05$). Both *T. terrestris* (100 and 200 mg/kg/day) and metformin reversed these changes: FSH, estradiol, and progesterone rose and LH fell relative to letrozole, with several pairwise differences significant (Tukey $p < 0.05$). Overall, *T. terrestris* 200 mg/kg/day and metformin produced the strongest normalization, approaching control values.

Histopathology

Phase- 1 Induction of PCOS (DAY 1-21)

Normal Control: A transverse section (TS) of the ovary from control female rat displays a clearly defined connective tissue stroma, a distinct medullary region and follicles at multiple developmental stages, including a mature Graafian follicle (mGF). The corpus luteum (CL), granulosa cells (GCs), and oocyte (OC) appear normal in structure (H & E $\times 100$). (Plate 5.8.1)

Letrozole 1 mg/kg/day: The ovarian section from rat exposed Letrozole 1 mg/kg/day for 21 days exhibits

marked degenerative alterations in the follicles and stromal tissue, along with noticeable vacuolization in the medullary area. Degeneration was evident in both the cortical and medullary zones, accompanied by a rise in the number of corpus luteum (CL) (H & E $\times 100$). (Plate 5.8.1)

Phase 2 Treatment PCOS (DAY 22-62)

Tribulus terrestris 100 mg/kg/day: The ovarian section from *Tribulus terrestris* treated rat for 40 days reveals signs of recovery toward normal morphology. The theca follicle remains intact, with a well-preserved zona granulosa (ZG), clearly visible oocyte (O), and defined zona pellucida (H & E $\times 400$). (Plate 5.8.2)

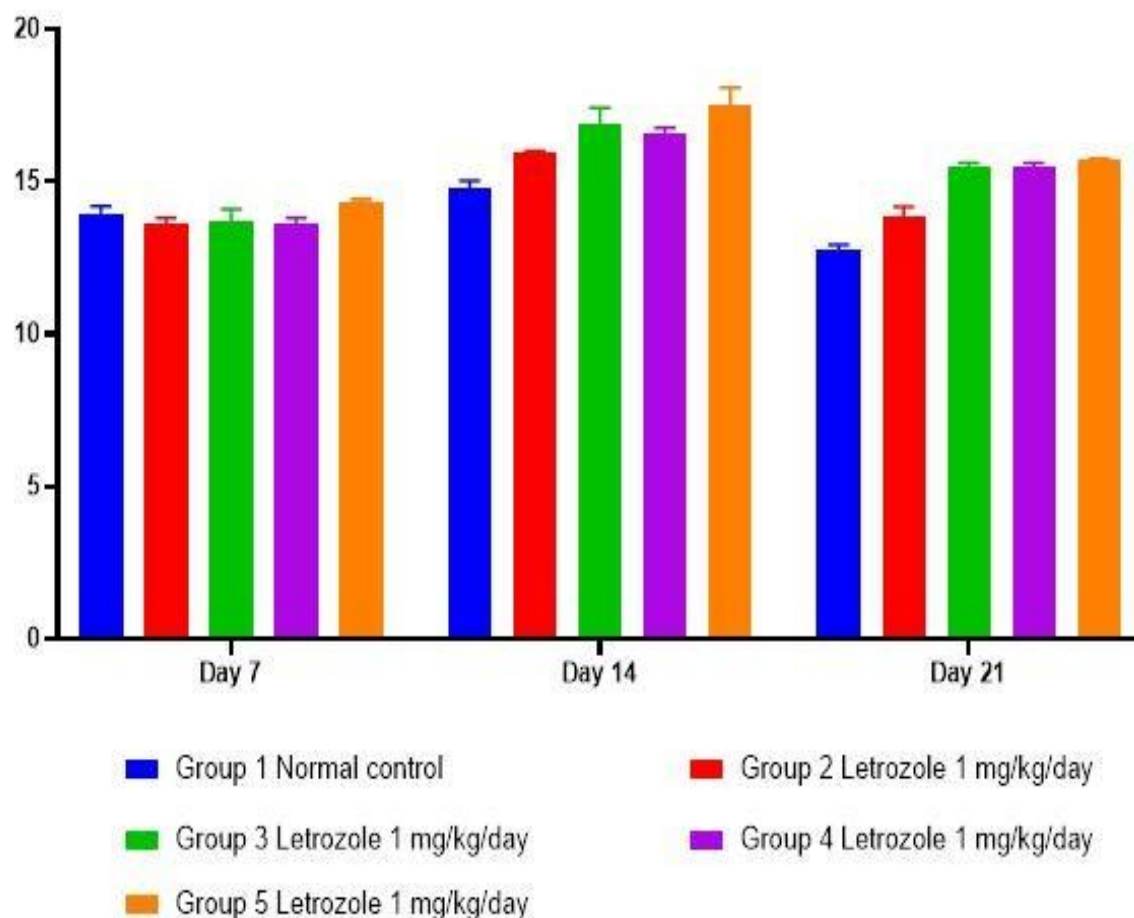
Tribulus terrestris 200 mg/kg/day: The ovarian section from *Tribulus terrestris* treated rat, following 40 days demonstrates signs of structural restoration. The theca follicle appears intact, with a normal zona granulosa (ZG), clearly visible oocyte (O), and a distinct zona pellucida (H & E $\times 400$). (Plate 5.8.2)

Table 5.2.1: Food Consumption During Letrozole-Induction Phase

LETROZOLE INDUCTION PHASE (21 days)					
Day	Group 1 Normal Control	Group 2 Letrozole 1 mg/kg/day	Group 3 Letrozole 1 mg/kg/day	Group 4 Letrozole 1 mg/kg/day	Group 5 Letrozole 1 mg/kg/day
Day 7	13.90 $\pm$ 0.29	13.66 $\pm$ 0.14	13.71 $\pm$ 0.38	13.66 $\pm$ 0.14	14.36 $\pm$ 0.07
Day 14	14.76 $\pm$ 0.28	15.95 $\pm$ 0.05	16.90 $\pm$ 0.52	16.57 $\pm$ 0.19	17.52 $\pm$ 0.57
Day 21	12.80 $\pm$ 0.14	13.85 $\pm$ 0.33	15.47 $\pm$ 0.14	15.47 $\pm$ 0.14	15.71 $\pm$ 0.04

Table 5.2.1: Shows the Mean daily food consumption (g/day) observed throughout the letrozole induction

Food Consumption During Letrozole-Induction Phase



phase. Data are articulated as Mean $\pm$ SEM. Asterisks (*) indicate significant differences the normal control group vs. letrozole treated at same timepoint ($p < 0.05$). Statistical comparisons were achieved using one-way ANOVA followed by Tukey's post hoc test.

Graph 5.2.1 Food Consumption of Rats During Letrozole-Induction Phase

Table 5.2.2: Food Consumption During Treatment Phase

TRIBULUS TERRESTRIS TREATMENT PHASE					
Day	Group 1 Normal Control	Group 2 PCOS Control	Group 3 <i>T. Terrestris</i> 100 mg/kg/day	Group 4 <i>T. Terrestris</i> 200 mg/kg/ day	Group 5 Metformin 300 mg/kg/day
Day 22	12.05 ± 1.68	11.76 ± 3.97	16.19 ± 0.54 **	11.14 ± 0.81	14.52 ± 0.67 *
Day 28	12.81 ± 3.57	14.88 ± 3.67	17.81 ± 0.14 **	17.29 ± 0.47 **	17.57 ± 0.20 **
Day 35	10.52 ± 2.27	11.81 ± 4.31	14.71 ± 0.22 *	13.90 ± 1.75 *	13.71 ± 0.93 *
Day 42	11.29 ± 3.16	11.40 ± 3.67	12.90 ± 0.15	12.48 ± 0.20	13.71 ± 0.67 *
Day 49	12.14 ± 1.14	11.52 ± 3.37	14.62 ± 0.61 *	16.10 ± 0.54 **	14.48 ± 1.55 *
Day 56	11.67 ± 3.84	12.23 ± 2.63	15.00 ± 0.47 **	14.35 ± 0.27 **	15.19 ± 0.61 **
Day 62	11.95 ± 3.25	12.48 ± 2.85	15.33 ± 0.40 **	14.62 ± 0.32 **	15.52 ± 0.70 **

Data were expressed as Mean ± SD, *p<0.05, **p<0.01

Table 5.2.2: Shows the Mean daily food consumption (g/day) observed throughout the Tribulus Terrestris Treatment Phase. Data are written as Mean ± SEM. Asterisks (*) indicate significant differences the normal control group vs. letrozole treated at the similar timepoint (p<0.05). Statistical comparisons were achieved using one-way ANOVA followed by Tukey's post hoc test.

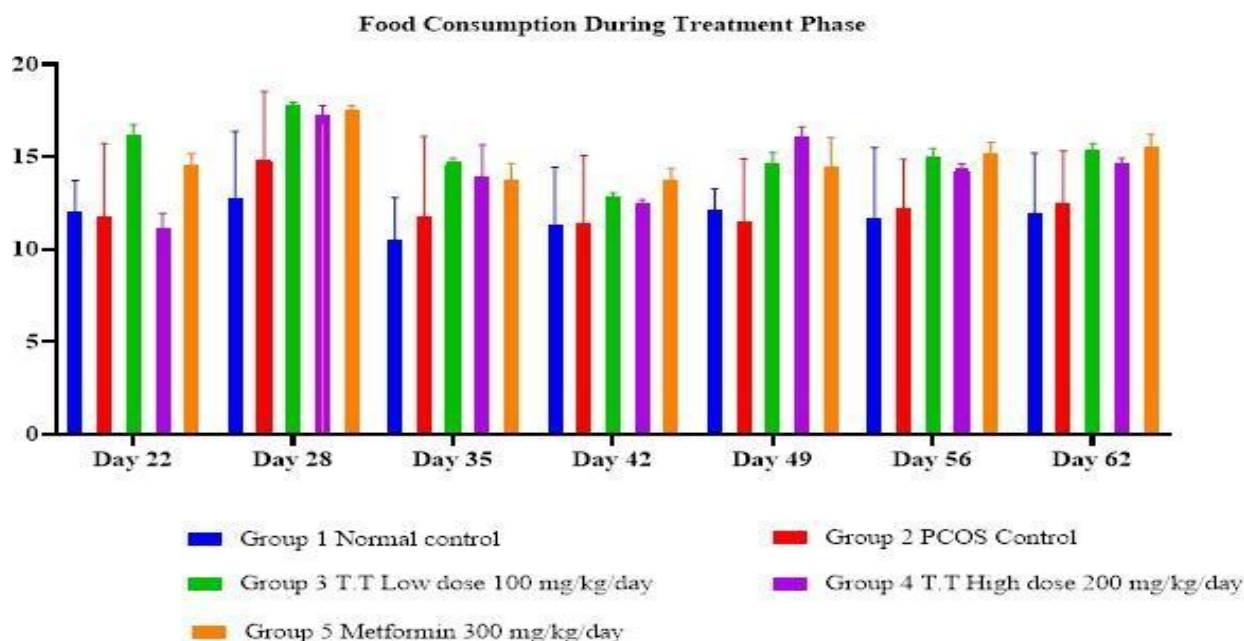
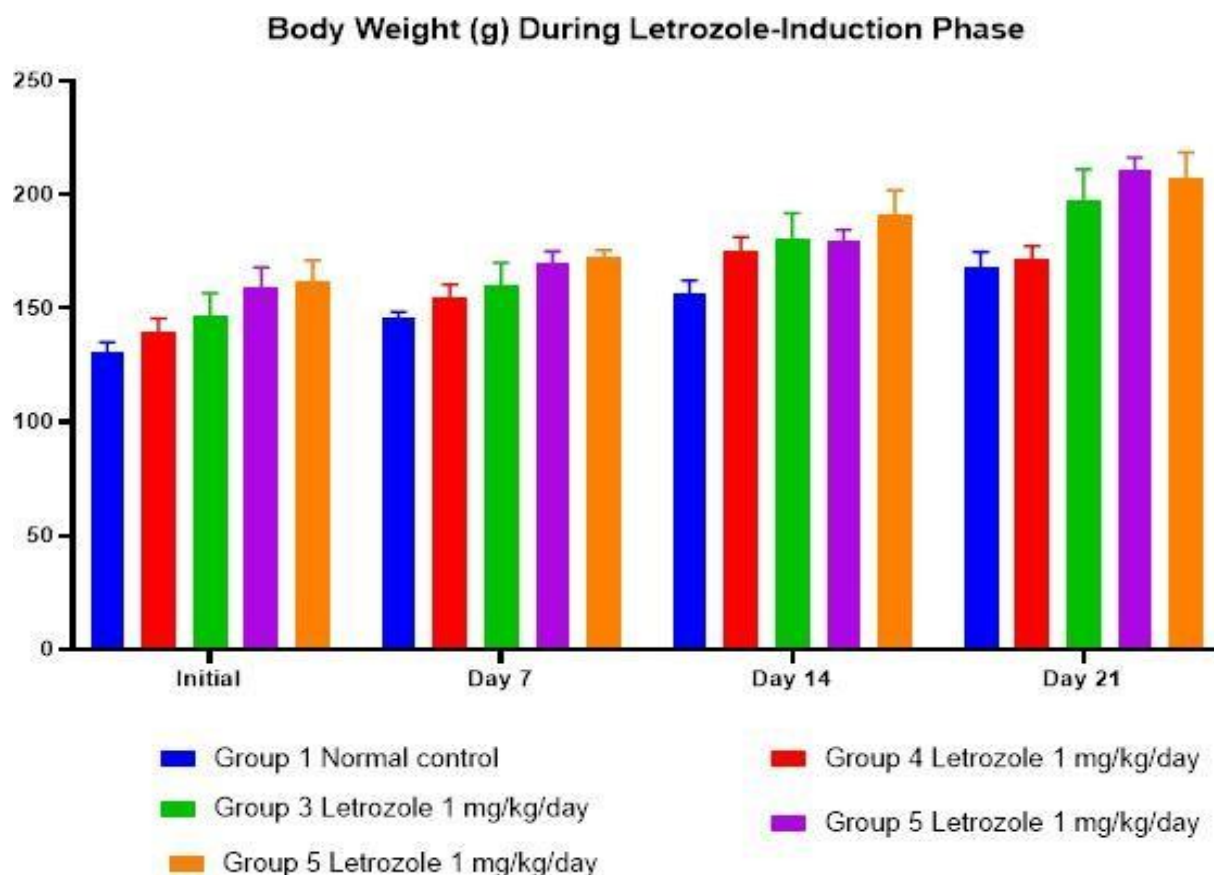


Table 5.3.1: Body Weight (g) During Letrozole-Induction Phase

LETROZOLE INDUCTION PHASE (21 days)					
Day	Group 1 Normal Control	Group 2 PCOS Control	Group 3 Letrozole 1 mg/kg/day	Group 4 Letrozole 1 mg/kg/day	Group 5 Letrozole 1 mg/kg/day

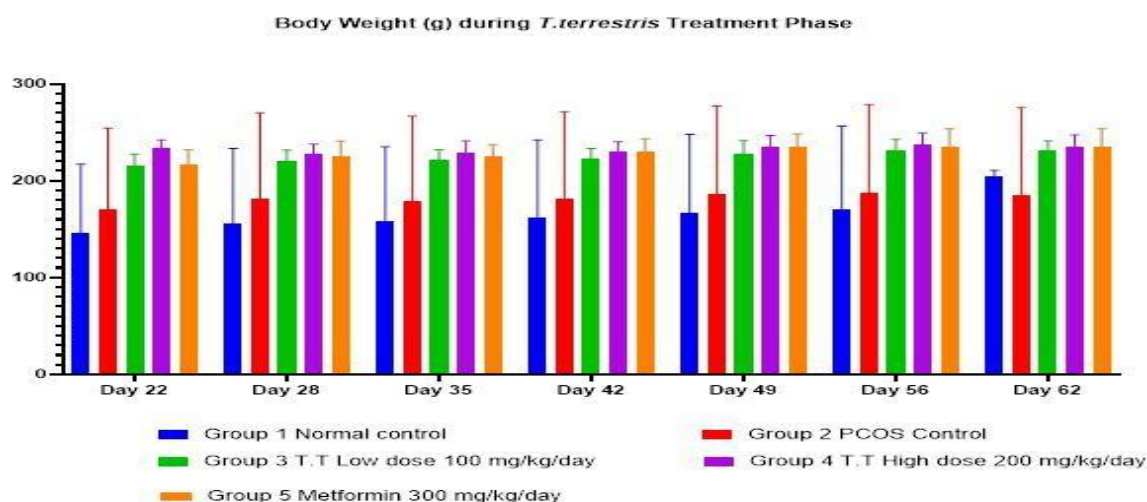
Initial	131.0 ± 3.9	139.7 ± 5.7	146.7 ± 9.9	159.3 ± 8.6	162.0 ± 9.1
Day 7	145.7 ± 2.7	154.7 ± 5.5	160.0 ± 10.0	169.8 ± 5.1	172.7 ± 2.6
Day 14	156.3 ± 5.7	175.3 ± 5.8	180.7 ± 10.9	179.7 ± 4.6	190.7 ± 10.9
Day 21	168.3 ± 6.3	171.3 ± 5.9	197.0 ± 14.1*	210.7 ± 5.5*	207.0 ± 11.4*



Graph 5.2.1 Body weight Trends across groups during Letrozole-Induction Phase

Table 5.3.2: Body Weight (g) During *T. terrestris* Treatment Phase

TRIBULUS TERRESTRIS TREATMENT PHASE					
Day	Group 1 Normal Control	Group 2 PCOS Control	Group 3 <i>T. Terrestris</i> 100 mg/kg/day	Group 4 <i>T. Terrestris</i> 200 mg/kg/day	Group 5 Metformin 300 mg/kg/day
Day 22	145.7 ± 71.5	170.7 ± 83.7	215.7 ± 12.0*	233.3 ± 8.9*	217.0 ± 15.3*
Day 28	156.3 ± 76.9	181.0 ± 89.2	220.7 ± 11.1*	228.3 ± 9.6*	225.0 ± 15.9*
Day 35	157.7 ± 77.5	179.0 ± 87.9	221.0 ± 10.9*	229.0 ± 12.2*	225.7 ± 11.6*
Day 42	162.3 ± 79.7	182.0 ± 89.3	222.3 ± 11.0*	230.3 ± 10.0*	230.3 ± 13.1*
Day 49	166.5 ± 81.6	186.0 ± 91.2	228.3 ± 13.2*	235.0 ± 11.7*	234.7 ± 13.7*
Day 56	170.7 ± 85.8	187.0 ± 91.7	231.3 ± 11.4*	237.0 ± 12.5*	235.3 ± 18.3*
Day 62	204.8 ± 6.1*	185.0 ± 90.7	231.3 ± 10.0*	235.0 ± 12.5*	235.3 ± 18.3*



Graph 5.3.2 Body weight Trends across groups during Treatment Phase Table 5.4.1: Estrous Cycle Pattern During Letrozole-Induction phase (Day 1–21)

Data show Mean $\pm$ SD number of days in every stage for every group (n = 6 animals). An asterisk denotes significant difference (t- test, $p < 0.05$) versus the normal control.

GRAPH 5.4.1 Dose-Dependent changes in estrous cycle in experimental groups Table 5.4.2: Estrous Cycle Pattern During Treatment phase (Days 22–62)

Data show Mean $\pm$ SD number of days in every stage for every group (n = 6 animals). An asterisk denotes significant difference (t- test, $p < 0.05$) versus the normal control.

Stage	Group 1 Normal Control	Group 2 Letrozole 1 mg/kg/day	Group 3 Letrozole 1 mg/kg/day	Group 4 Letrozole 1 mg/kg/day	Group 5 Letrozole 1 mg/kg/day
Proestrus	1.80 $\pm$ 0.84	1.20 $\pm$ 0.75	1.20 $\pm$ 0.82	1.20 $\pm$ 0.82	0.60 $\pm$ 0.55*

Stage	Group 1 Normal Control	Group 2 PCOS Control	Group 3 <i>T. terrestris</i> 100 mg/kg/day	Group 4 <i>T. terrestris</i> 200 mg/kg/day	Group 5 Metformin 300 mg/kg/day
Proestrus	3.0 $\pm$ 1.2	0.0 $\pm$ 0.0*	2.2 $\pm$ 0.4	3.2 $\pm$ 0.4	2.8 $\pm$ 0.4
Estrus	5.4 $\pm$ 1.1	0.0 $\pm$ 0.0*	6.4 $\pm$ 0.9	7.2 $\pm$ 1.1*	6.6 $\pm$ 0.5
Metestrus	3.2 $\pm$ 1.1	0.0 $\pm$ 0.0*	3.4 $\pm$ 0.5	2.8 $\pm$ 0.8	3.0 $\pm$ 0.7
Diestrus	9.0 $\pm$ 1.4	16.8 $\pm$ 9.4	9.0 $\pm$ 1.2	7.6 $\pm$ 1.1	8.2 $\pm$ 1.3

Metestrus	2.60 $\pm$ 1.14	0.80 $\pm$ 1.17*	0.40 $\pm$ 0.52*	0.60 $\pm$ 0.52*	0.60 $\pm$ 0.55*
Diestrus	11.40 $\pm$ 2.07	17.20 $\pm$ 1.94*	16.80 $\pm$ 1.94*	17.00 $\pm$ 1.72*	18.20 $\pm$ 1.64*

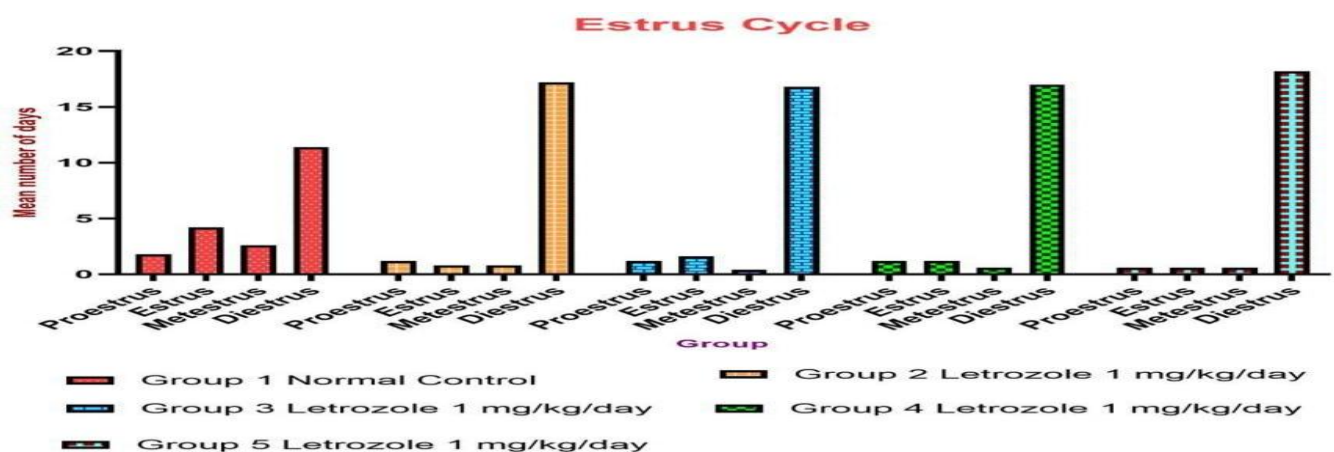


Table 5.5.1: Effect of *T. terrestris* extract on organ weights in treatment rats (Mean $\pm$ SD).

Organ	Group 1 Normal Control (Mean $\pm$ SD)	Group 2 PCOS Control (Mean $\pm$ SD)	Group 3 <i>T. Terrestris</i> 100 mg/kg/day (Mean $\pm$ SD)	Group 4 <i>T. Terrestris</i> 200 mg/kg/day (Mean $\pm$ SD)	Group 5 Metformin 300 mg/kg/day (Mean $\pm$ SD)
Adrenal	0.091 $\pm$ 0.033	0.063 $\pm$ 0.016	0.077 $\pm$ 0.019	0.060 $\pm$ 0.028	0.091 $\pm$ 0.021
Brain	1.652 $\pm$ 0.104	1.702 $\pm$ 0.042	1.372 $\pm$ 0.169	1.595 $\pm$ 0.138	1.476 $\pm$ 0.233
Heart	0.956 $\pm$ 0.407	0.788 $\pm$ 0.088	0.661 $\pm$ 0.237	0.804 $\pm$ 0.113	0.655 $\pm$ 0.223
Kidney	1.862 $\pm$ 0.510	1.819 $\pm$ 0.133	1.700 $\pm$ 0.168	1.683 $\pm$ 0.237	1.667 $\pm$ 0.169
Liver	7.181 $\pm$ 0.858	7.088 $\pm$ 1.013	6.847 $\pm$ 1.001	6.481 $\pm$ 0.189	7.222 $\pm$ 1.339
Ovary	0.154 $\pm$ 0.047	0.166 $\pm$ 0.073	0.282 $\pm$ 0.285	0.156 $\pm$ 0.049	0.452 $\pm$ 0.299
Spleen	0.718 $\pm$ 0.069	0.677 $\pm$ 0.050	0.722 $\pm$ 0.044	0.337 $\pm$ 0.268	0.699 $\pm$ 0.040
Thymus	0.294 $\pm$ 0.121	0.365 $\pm$ 0.214	0.233 $\pm$ 0.091	0.332 $\pm$ 0.202	0.244 $\pm$ 0.029
Thyroid	0.222 $\pm$ 0.128	0.119 $\pm$ 0.017	0.199 $\pm$ 0.075	0.214 $\pm$ 0.074	0.172 $\pm$ 0.051
Uterus	0.621 $\pm$ 0.452	0.386 $\pm$ 0.108	0.466 $\pm$ 0.144	0.444 $\pm$ 0.165	0.321 $\pm$ 0.050

Data obtained written as Mean $\pm$ SD (6 per group shown in the raw data). P-values and significance markers (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant) reflect comparisons versus Group 1.

ESTROUS CYCLE

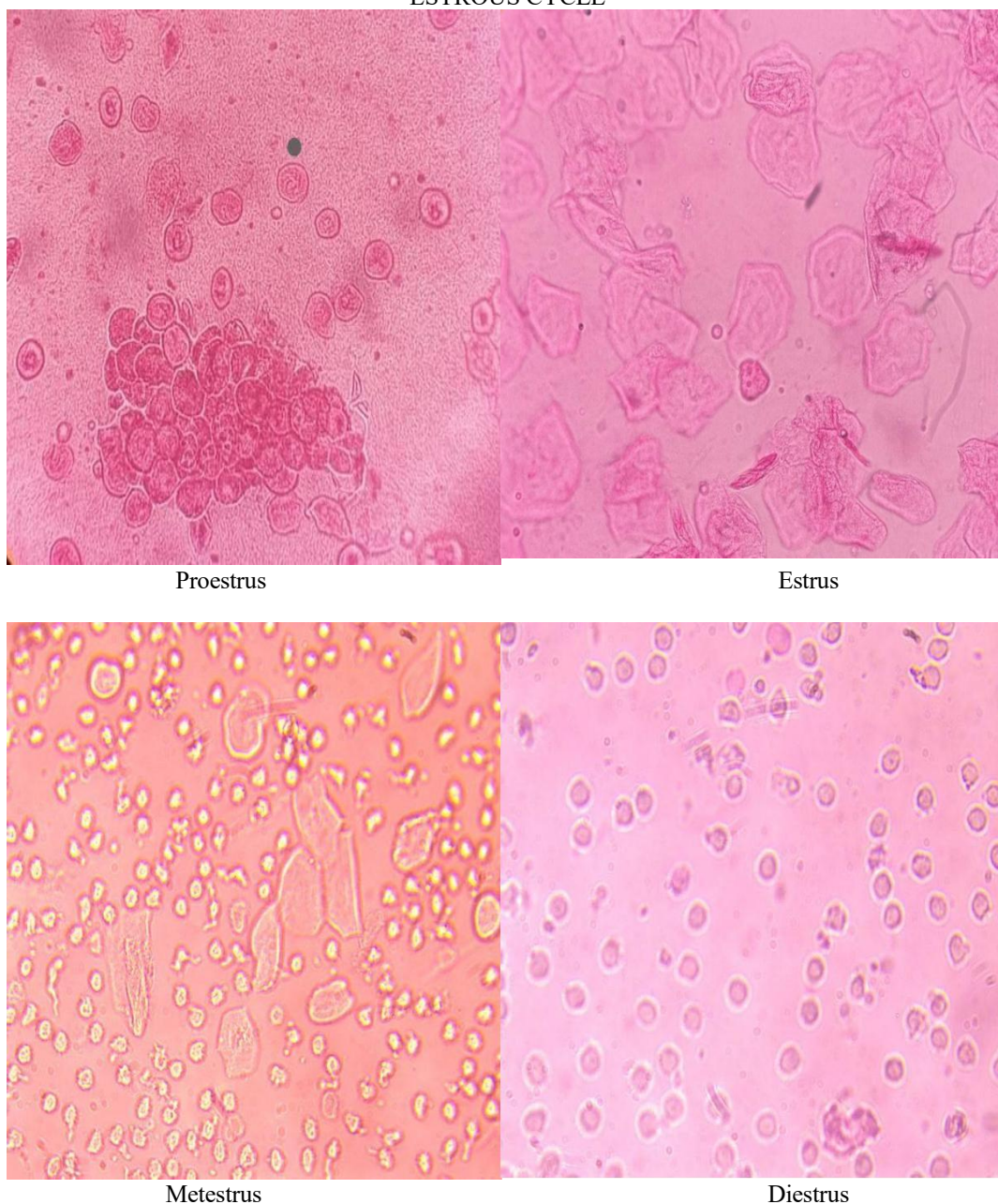


Plate 5.4.1 Phases of Estrous cycle identified from vaginal smear (Stained and observed under Binocular Microscope).

Table 5.5.2: Statistical comparison of organ weights across treatment groups in experimental rats.

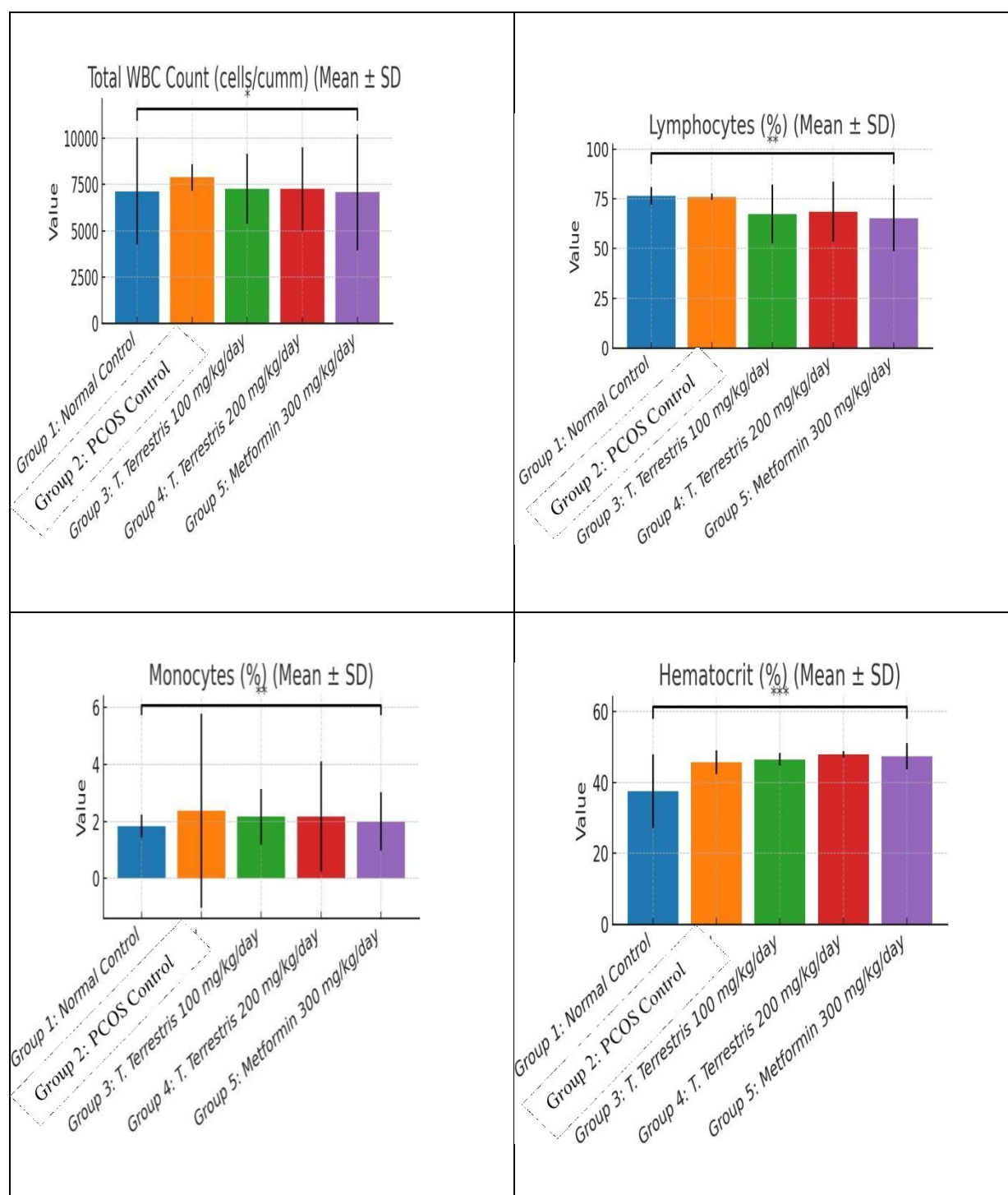
Organs	p vs Control Group 2 PCOS Control	p vs Control Group 3 T. Terrestris 100 mg/kg/day	p vs Control Group 4 T. Terrestris 200 mg/kg/day	p vs Control Group 5 Metformin 300 mg/kg/day
Adrenal	0.1041 ns	0.3798 ns	0.1446 ns	1.0000 ns
Brain	0.3177 ns	0.0081 **	0.5145 ns	0.1358 ns
Heart	0.3656 ns	0.1633 ns	0.4197 ns	0.1519 ns
Kidney	0.8467 ns	0.4884 ns	0.4770 ns	0.4078 ns
Liver	0.8666 ns	0.5487 ns	0.1040 ns	0.9515 ns

Ovary	0.7648 ns	0.3249 ns	0.9608 ns	0.0583 ns
Spleen	0.2815 ns	0.9146 ns	0.0623 ns	0.5791 ns
Thymus	0.5323 ns	0.3464 ns	0.7512 ns	0.3648 ns
Thyroid	0.1084 ns	0.7167 ns	0.9158 ns	0.4130 ns
Uterus	0.2664 ns	0.4561 ns	0.4113 ns	0.1665 ns

Data obtained were written as Mean $\pm$ SD (6 per group shown in the raw data). P-values and significance markers (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant) reflect comparisons versus Group 1.

Table 5.6.1: Hematological parameters (Mean $\pm$ SD) of control and experimental groups.

Parameter	Group 1 Normal Control	Group 2 PCOS Control	Group 3 T. Terrestris 100 mg/kg/day	Group 4 T. Terrestris 200 mg/kg/day	Group 5 Metformin 300 mg/kg/day
Total WBC Count (cells/cumm)	7142.00 $\pm$ 2889.95 *	7886.67 $\pm$ 722.26 *	7268.33 $\pm$ 1884.17 *	7253.33 $\pm$ 2255.98 *	7086.00 $\pm$ 3143.89 *
Neutrophils (%)	19.50 $\pm$ 3.89	21.33 $\pm$ 2.42	27.17 $\pm$ 12.66	26.33 $\pm$ 12.18	32.00 $\pm$ 15.68
Lymphocytes (%)	76.50 $\pm$ 4.37 **	76.00 $\pm$ 1.67 **	67.33 $\pm$ 14.80 **	68.50 $\pm$ 15.16 **	65.20 $\pm$ 16.62 **
Eosinophils (%)	1.50 $\pm$ 0.55	1.67 $\pm$ 0.52	2.50 $\pm$ 1.76	2.50 $\pm$ 1.38	2.60 $\pm$ 1.37
Monocytes (%)	1.83 $\pm$ 0.41 **	2.37 $\pm$ 3.41 **	2.17 $\pm$ 0.98 **	2.17 $\pm$ 1.94 **	2.00 $\pm$ 1.03 **
RBCs (mil/cumm)	7.17 $\pm$ 2.04	9.83 $\pm$ 2.81	8.87 $\pm$ 0.70	9.38 $\pm$ 0.48	9.94 $\pm$ 0.21
Hemoglobin (gm%)	12.45 $\pm$ 3.13	14.33 $\pm$ 1.98	19.38 $\pm$ 10.70	15.53 $\pm$ 0.49	14.86 $\pm$ 1.56
Hematocrit (%)	37.57 $\pm$ 10.42 ***	45.78 $\pm$ 3.31 ***	46.52 $\pm$ 1.79 ***	47.97 $\pm$ 0.86 ***	47.42 $\pm$ 3.77 ***
MCV (fL)	51.18 $\pm$ 0.72	45.33 $\pm$ 15.70	48.87 $\pm$ 4.13	50.12 $\pm$ 0.72	50.86 $\pm$ 0.59
MCH (pg)	17.15 $\pm$ 0.91	19.77 $\pm$ 6.22	17.15 $\pm$ 1.17	49.28 $\pm$ 81.98	16.56 $\pm$ 0.25
MCHC (gm/dl)	33.20 $\pm$ 1.70	28.58 $\pm$ 9.21	32.52 $\pm$ 0.70	32.72 $\pm$ 0.94	32.66 $\pm$ 1.27
Platelets (lakhs/cumm)	9.47 $\pm$ 3.97	9.88 $\pm$ 1.75	10.28 $\pm$ 1.24	9.75 $\pm$ 0.35	9.88 $\pm$ 0.56
MPV (fL)	8.12 $\pm$ 0.24	7.98 $\pm$ 0.44	8.38 $\pm$ 0.47	7.78 $\pm$ 0.57	7.72 $\pm$ 0.32

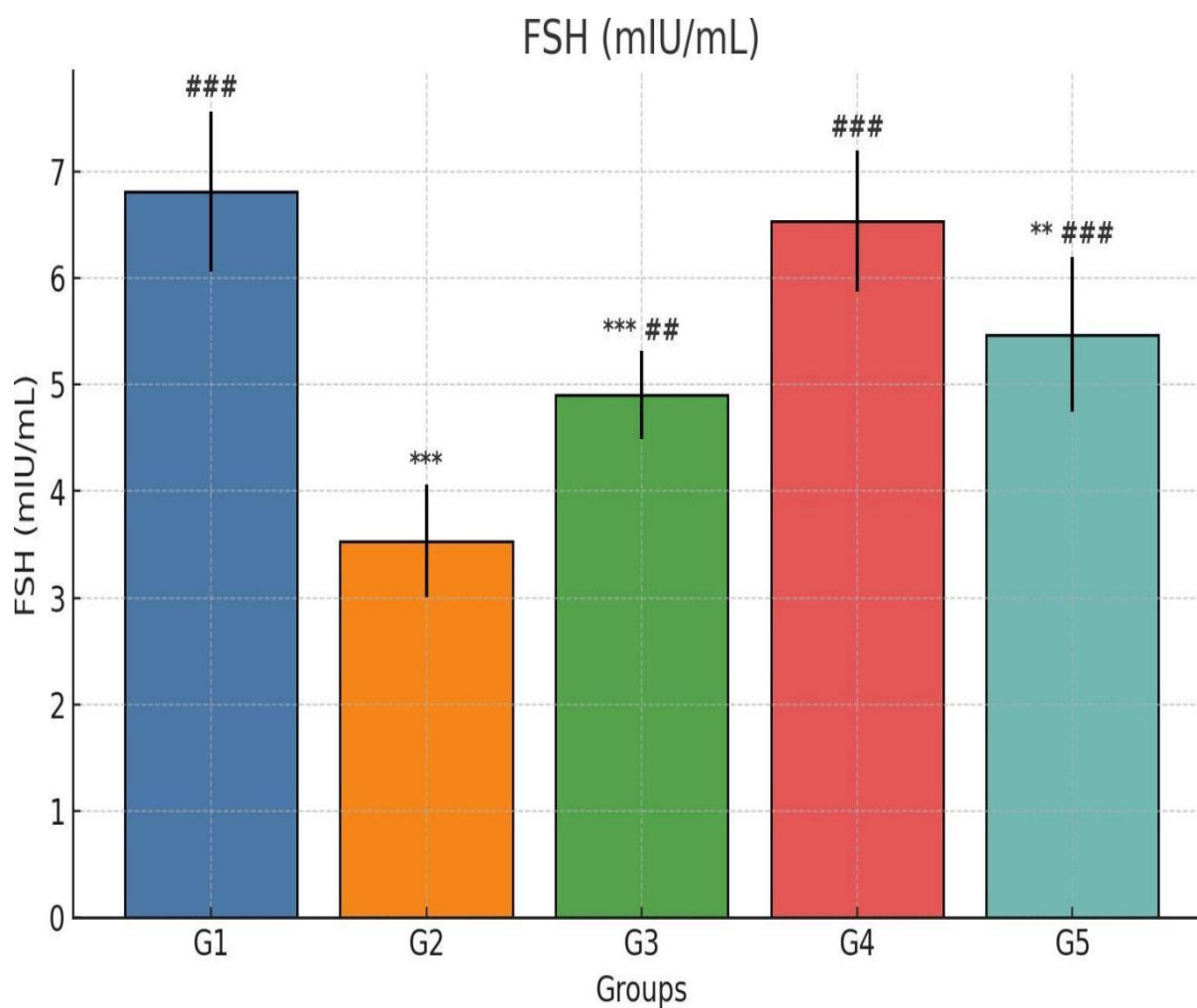
**GRAPH 5.6.1: Dose-Dependent changes in Hematological Parameters During Treatment Phase****TABLE 5.7.1: Serum FSH, LH, Estradiol, and Progesterone Levels Across Experimental Groups**

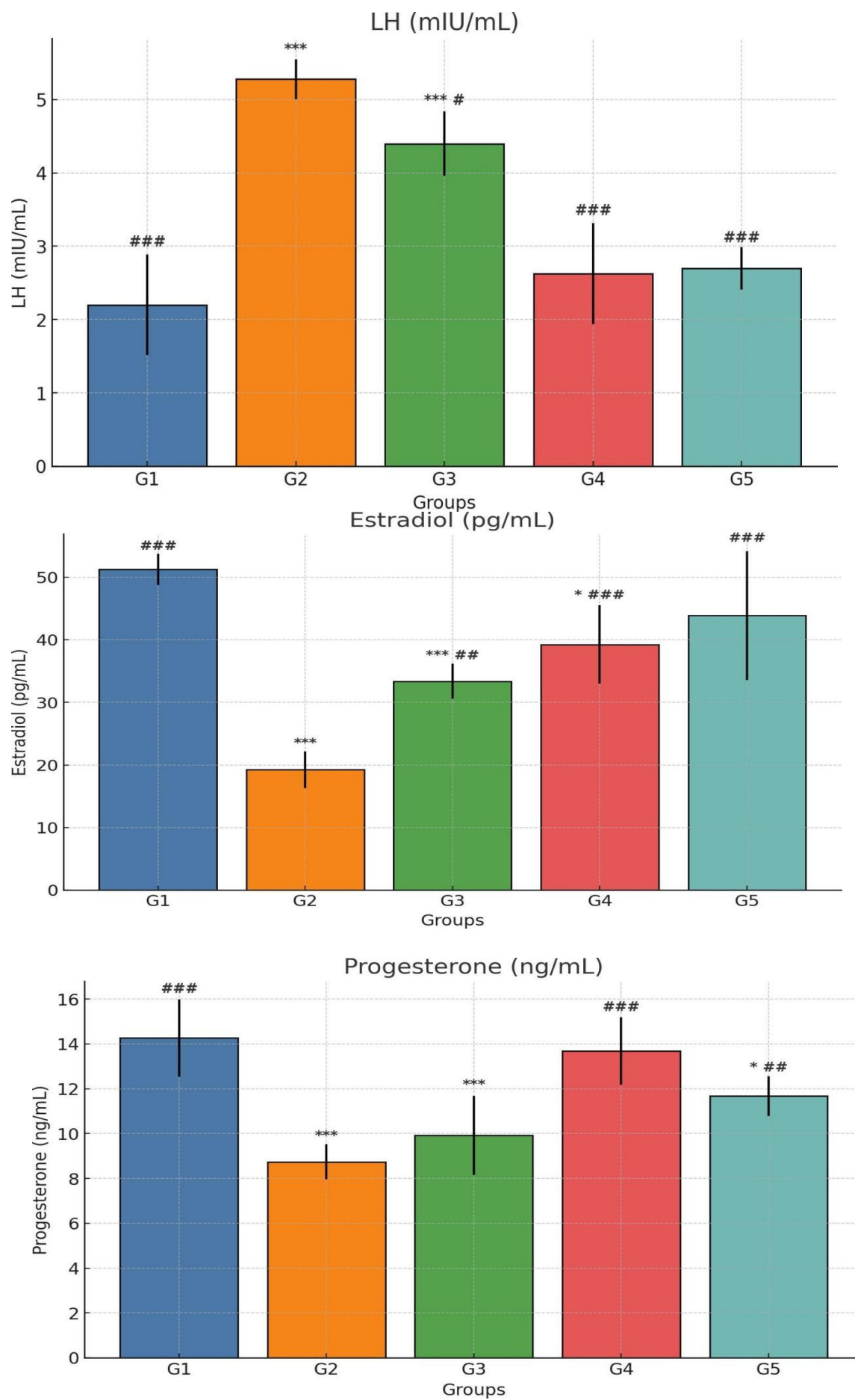
Group	FSH (mIU/mL)	LH (mIU/mL)	Estradiol (pg/mL)	Progesterone (ng/mL)
Group 1 Normal Control	6.81 $\pm$ 0.75	2.20 $\pm$ 0.68	51.27 $\pm$ 2.49	14.27 $\pm$ 1.72
Group 2 PCOS Control	3.53 $\pm$ 0.53	5.28 $\pm$ 0.27	19.24 $\pm$ 2.91	8.74 $\pm$ 0.78

Group 3 T.terrestris 100 mg/kg/day	4.90 ± 0.41	4.40 ± 0.44	33.39 ± 2.80	9.92 ± 1.77
Group 4 T.terrestris 200 mg/kg/day	6.53 ± 0.66	2.63 ± 0.69	39.24 ± 6.26	13.68 ± 1.51
Group 5 Metformin 300 mg/kg/day	5.47 ± 0.73	2.70 ± 0.29	43.88 ± 10.32	11.68 ± 0.89

Table 5.7.2: ANOVA Summary

Analyte	F	p-value
FSH (mIU/mL)	26.46	1.15e-08
LH (mIU/mL)	40.84	1.29e-10
Estradiol (pg/mL)	26.03	1.35e-08
Progesterone (ng/mL)	17.24	6.62e-07







GRAPH 5.7.1: Concentration of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Estradiol, Progesterone Across Experimental Groups

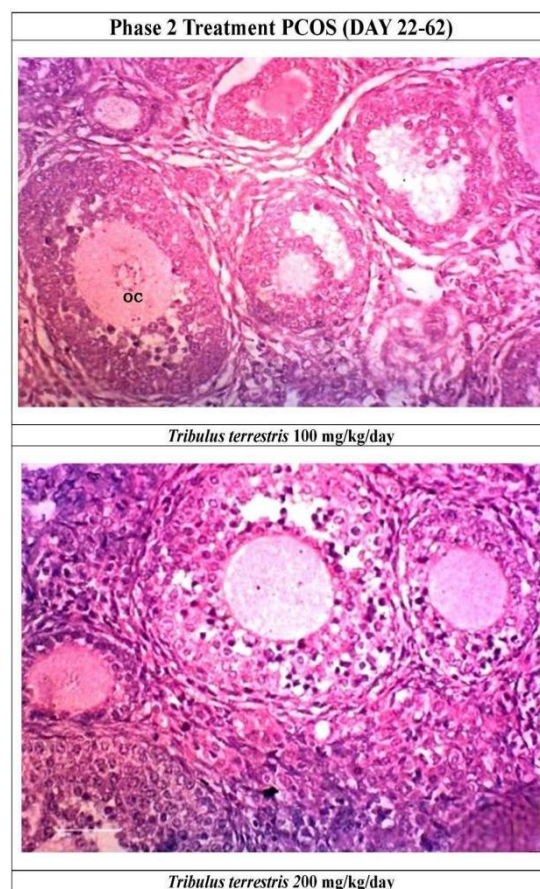
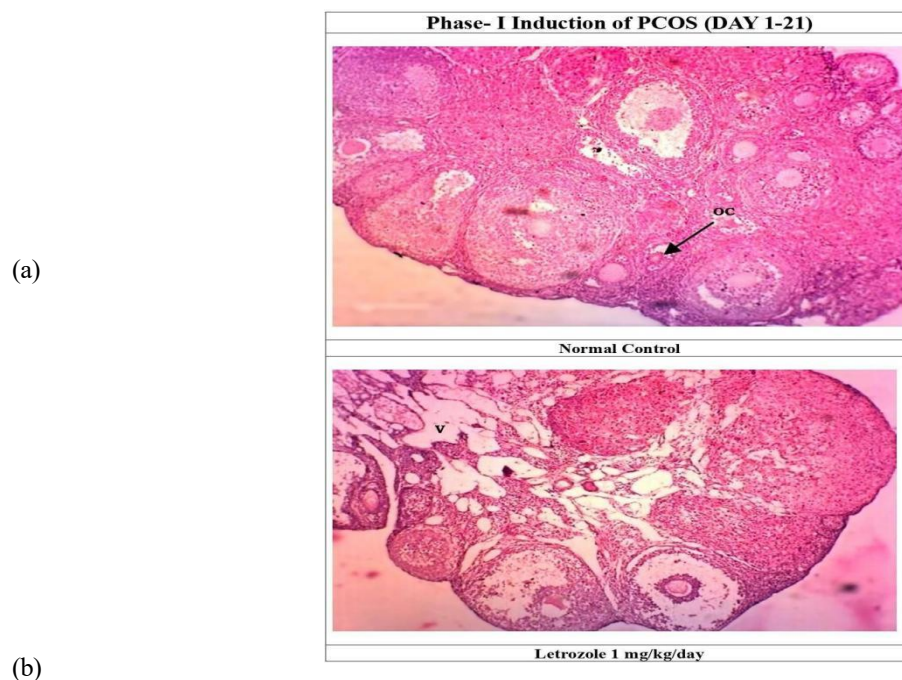


Plate 5.8.1 Photomicrographic Representation of Ovarian Histopathology in Normal Control and Letrozole induced PCOS Groups During PCOS Induction Phase (Day1-21)

(a)OC – indicates Oocyte **(b)** V – indicates Vacuolization

Plate 5.8.2 Photomicrographic Representation of Ovarian Histopathology in Tribulus

terrestris 100 mg/kg/day and Tribulus terrestris 200 mg/kg/day During Treatment Phase (Day 22-62) (c)
OC – Indicates Oocyte.

Discussion

The present study demonstrated that *Tribulus terrestris* extract effectively ameliorated endocrine, metabolic, and reproductive disturbances in letrozole-induced PCOS rats. Induction with letrozole produced persistent diestrus, reduced estrus/metestrus phases, and increased body weight, findings reliable with previous reports that aromatase inhibition leads to hyperandrogenism and anovulation [40],[6]. In our experiment, treatment with *T. terrestris* significantly restored estrous cyclicity, with the high dose (200 mg/kg) producing estrus days even greater than controls, corroborating previous experiments showing that *T. terrestris* induces corpus luteum formation and normalizes ovarian activity [13],[56].

Hormonal assays revealed that letrozole reduced FSH, estradiol, and progesterone while elevating LH, consistent with the PCOS phenotype described by Rosenfield and Ehrmann (2016) [53]. Administration of *T. terrestris* reversed these disruptions, especially at 200 mg/kg, where hormonal profiles closely approached normal controls. These outcomes align with earlier findings that phytoestrogens and protodioscin in *T. terrestris* act on estrogen receptors to regulate gonadotropin secretion and promote ovulation [26],[48]. Similarly, the observed effects of metformin in normalizing hormone levels and estrous cycles showing consistent with previous work highlighting its role in enhancing insulin sensitivity and reducing androgen production in PCOS [46],[44]. Notably, *T. terrestris* exhibited comparable efficacy to metformin, supporting its potential as a natural therapeutic alternative.

Hematological parameters further emphasized the systemic impact of letrozole, which elevated leukocyte counts and hematocrit, indicating inflammatory and stress responses. Treatment with *T. terrestris* normalized these alterations, reflecting its immunomodulatory and antioxidant activities, consistent with prior reports of its free radical scavenging and anti-inflammatory potential [57]. Body weight and food consumption trends also supported the therapeutic efficacy of *T. terrestris*. While letrozole led to abnormal weight gain and reduced feeding, both *T. terrestris* and metformin reversed these changes, findings with that of prior observations to herbal extracts improve metabolic balance in PCOS [70].

Histopathological analysis confirmed structural ovarian recovery with *T. terrestris*, where normal follicular development, intact zona granulosa, and reduced cystic degeneration were evident. These results mirror reports by Esfandiari et al. (2011) [19] and Dehghan et al. (2012)

[13] that *T. terrestris* extract restores ovarian architecture by promoting follicular maturation and luteinization. Collectively, our findings strengthen the evidence that *T. terrestris* exerts multi-targeted actions—normalizing hormone levels, enhancing ovulatory cycles, modulating hematological balance, and reversing ovarian pathology.

Although both *T. terrestris* doses showed beneficial effects, the higher dose consistently demonstrated superior restoration across parameters, comparable to metformin. This dose-dependence suggests that the pharmacological activity of steroidal saponins and phytoestrogens increases with concentration, as reported previously [22],[41].

Conclusion

This study delivers the experimental proof that *Tribulus terrestris* extract alleviates reproductive and metabolic disturbances in letrozole-induced PCOS rats. The extract restored estrous cyclicity, normalized gonadotropin and steroid hormone profiles, improved hematological indices, and reversed ovarian histopathology, with efficacy comparable to metformin. These findings support the potential of *T. terrestris* as a phytotherapeutic candidate for managing PCOS, particularly as a safer alternative to synthetic drugs. Future investigations should focus on molecular pathways, dose optimization, and clinical trials to confirm its therapeutic applicability in women with PCOS.

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