



Therapeutic Effects of Marjoram (*Origanum majorana* L.) Extract and Nano-Formulation on Hormonal Parameters and Ovarian Histology in Letrozole-Induced Polycystic Ovary Syndrome in Rats

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Abstract: Polycystic ovary syndrome (PCOS) represents a prevalent endocrine disorder characterized by hyperandrogenism, hormonal imbalances, and ovarian dysfunction, affecting approximately 30% of female infertility cases globally. This study evaluated the therapeutic efficacy of marjoram (*Origanum majorana* L.) aqueous extract and zinc oxide nano-loaded marjoram formulation on letrozole-induced PCOS in female Wistar rats. Fifty rats (150-250 g) were randomly assigned to five groups (n=10): negative control (physiological saline), carboxymethylcellulose (CMC) control, PCOS induction (1 mg/kg letrozole for 58 days), marjoram extract treatment (45 mg/kg for 30 days post-induction), and nano-marjoram treatment (45 mg/kg for 30 days post-induction). Serum hormonal parameters and ovarian histology were assessed. Letrozole induction produced characteristic PCOS features with significant hormonal alterations: 44% FSH decrease (5.78 vs. 10.30 mIU/mL, $p<0.05$), 46% LH increase (20.07 vs. 13.71 mIU/mL, $p<0.05$), 43% progesterone decrease (6.87 vs. 12.07 mIU/mL, $p<0.05$), 32% estradiol decrease (72.91 vs. 106.66 pg/mL, $p<0.05$), 125% testosterone increase (0.45 vs. 0.20 pg/mL, $p<0.05$), and 158% inhibin-B increase (378.59 vs. 146.58 pg/mL, $p<0.05$) compared to controls. Nano-marjoram treatment demonstrated superior therapeutic effects, restoring FSH by 51% (8.73 mIU/mL), reducing LH by 18% (16.44 mIU/mL), increasing progesterone by 36% (9.33 mIU/mL), increasing estradiol by 24% (90.64 pg/mL), reducing testosterone by 42% (0.26 pg/mL), and reducing inhibin-B by 53% (179.35 pg/mL) relative to PCOS group. Histological examination revealed that nano-marjoram treatment significantly improved ovarian architecture, increasing the number of corpora lutea, restoring Graafian follicles, and reducing cystic follicles and inflammatory infiltration compared with the PCOS group. These findings demonstrate that marjoram, particularly in nano-formulation, effectively ameliorates PCOS-associated hormonal dysregulation and ovarian pathology through antioxidant and anti-androgenic mechanisms, suggesting potential clinical applications for PCOS management.

Keywords: Polycystic ovary syndrome; PCOS; marjoram; *Origanum majorana*; nanotechnology

1. Introduction

Polycystic ovary syndrome (PCOS) constitutes one of the most prevalent endocrine disorders affecting reproductive-aged women, with multisystemic metabolic manifestations extending beyond reproductive dysfunction [1]. First described in 1935 by American gynecologists Irving F. Stein Sr. and Michael L. Leventhal (subsequently termed Stein-Leventhal syndrome), PCOS represents a complex condition encompassing reproductive, metabolic, and psychological

dimensions [2]. The syndrome is characterized by dysregulation of reproductive hormone secretion, particularly elevated androgen levels, in conjunction with insulin resistance and increased obesity prevalence [3]. PCOS contributes to approximately 30% of female infertility cases globally, representing a substantial reproductive health burden [4]. Morphologically, PCOS-affected ovaries exhibit numerous small follicular cysts (2-10 mm diameter) presenting a characteristic pearl-like appearance [5]. Clinical manifestations include infertility, menstrual irregularities, alopecia, and hirsutism [6-8]. Conventional pharmacological interventions often produce adverse effects, prompting increased interest in natural product-derived therapeutics with improved safety profiles [9, 10].

Medicinal plants contain diverse bioactive compounds that are crucial for promoting human health [11]. These phytochemicals—including terpenoids, alkaloids, and polyphenols—exert antioxidant and anti-inflammatory effects by protecting against cellular oxidative stress and modulating the inflammatory response [12, 13]. Marjoram (*Origanum majorana* L.), a member of the Lamiaceae family, contains particularly abundant phenolic compounds, including phenolic acids and flavonoids, as well as potent antioxidants such as carvacrol [14]. Rosmarinic acid, identified as one of marjoram's most powerful polyphenolic antioxidants, contributes significantly to its therapeutic potential, which explains its widespread traditional medicinal use [15]. Recent advances in nanotechnology have enabled the development of nano-formulated herbal therapeutics with enhanced bioavailability and therapeutic efficacy [16]. Nano-delivery systems can improve pharmacokinetic properties, cellular uptake, and target tissue distribution of bioactive compounds [17]. Despite marjoram's documented antioxidant and hormonal regulatory properties, comprehensive quantitative evaluations of marjoram extract and nano-formulations in PCOS treatment remain limited. This study aimed to quantitatively evaluate the therapeutic efficacy of marjoram aqueous extract and zinc oxide nano-loaded marjoram formulation on hormonal parameters and ovarian histology in letrozole-induced PCOS rat models, providing evidence-based insights for potential PCOS therapeutic applications.

2. Materials and Methods

2.1 Ethical Approval

All experimental procedures were conducted in accordance with guidelines approved by the Animal Housing Committee, University of Karbala, Faculty of Pharmacy, and under the supervision of the Department of Life Sciences, College of Education, Al-Qadisiyah University. Animal care and handling protocols adhered to international standards for laboratory animal welfare.

2.2 Experimental Animals and Housing Conditions

Fifty female Wistar rats weighing 150-250 g were obtained from the Faculty of Pharmacy, University of Karbala, Iraq. Animals were acclimated for one week before experimental procedures. Housing conditions were maintained at controlled temperature (21-22°C), with 12-hour light/dark cycles and *ad libitum* access to standard rodent chow and water.

2.3 Experimental Design and Treatment Groups

Animals were randomly assigned to five experimental groups (n=10 per group): Group 1 (G1, Negative Control): Administered physiological saline (0.9% NaCl) orally for 58 days. Group 2 (G2, CMC Control): Administered 1% carboxymethylcellulose (CMC) solution orally for 58 days, followed by physiological saline for 30 days (total 58 days). Group 3 (G3, PCOS Induction): Administered letrozole (1 mg/kg body weight) dissolved in 1% CMC solution orally for 58 days to induce PCOS [18], followed by physiological saline for 30 days. Group 4 (G4, Marjoram Extract Treatment): Administered letrozole (1 mg/kg) in 1% CMC for 58 days, followed by marjoram aqueous extract (45 mg/kg body weight) orally for 30 days [19]. Group 5 (G5, Nano-Marjoram Treatment): Administered letrozole (1 mg/kg) in CMC for 58 days, followed by zinc oxide nano-loaded marjoram formulation (45 mg/kg body weight) orally for 30 days [20].

2.4 PCOS Induction Protocol

PCOS was induced by daily oral administration of letrozole (1 mg/kg body weight) dissolved in 0.5% CMC solution for 58 consecutive days [21]. Letrozole, a non-steroidal aromatase inhibitor, blocks estrogen synthesis from androgens, resulting in hyperandrogenism and ovarian dysfunction characteristic of PCOS.

Vaginal cytology was performed during the 58-day letrozole induction period. Smears were collected every three days and examined microscopically. All animals assigned to PCOS-induced groups (G3, G4, G5) demonstrated persistent estrus (predominantly cornified epithelial cells) and disrupted estrous cyclicity by day 21, confirming successful PCOS induction before treatment initiation. Regarding the choice of letrozole-only protocol without high-fat diet: this design was intentionally selected to isolate the androgenic/hormonal component of PCOS, which was the primary focus of the study.

2.5 Marjoram Aqueous Extract Preparation

Marjoram (*Origanum majorana* L.) leaves were procured from the herbal market, Shorja district, Baghdad Governorate, Iraq (March 2024). Plant material was botanically authenticated and stored in clean plastic containers away from heat, humidity, and light. An aqueous extract was prepared according to established protocols [22]. Briefly, 1 kg of pulverized, dried leaves was soaked in 10 L of distilled water (1:10 w/v) for 72 hours at room temperature (cold extraction method). The mixture was subsequently heated on a magnetic stirrer hot plate at 25-30°C for 4 hours, filtered through Whatman No. 1 filter paper, and concentrated. The extract was stored in opaque, sealed glass bottles at 4°C and utilized within three months of preparation.

2.6 Zinc Oxide Nano-Marjoram Formulation Preparation

Nano-formulation was synthesized following the modified procedures of Bashi et al. [23]. Briefly, 1 g of marjoram aqueous extract was added to a zinc oxide solution (1 g ZnO dissolved in 50 mL deionized distilled water), and the mixture was incubated at room temperature for 24 hours. Particle size (DLS: 45.3 +/- 3.2 nm), polydispersity index (PDI: 0.18 +/- 0.02), zeta potential (-22.4 +/- 1.8 mV), and TEM morphology confirming spherical nanoparticle formation. These data confirm that nanoparticles were successfully formed and are colloiddally stable. The ZnO content of the administered dose has been calculated (approximately 8.5 mg/kg), which is well below established rodent toxicity thresholds (LD50 >240 mg/kg). The mixture was subsequently placed in a shaker incubator at 40°C for 18 hours. A color change from pale yellow to reddish yellow indicated nanoparticle formation. The precipitate was separated by centrifugation (3000 rpm, 20 minutes), washed repeatedly with deionized water, dried in an electric furnace at 50°C, pulverized using a ceramic mortar, and stored at 4°C until use.

2.7 Sample Collection and Processing

At experimental termination (day 58), animals were anesthetized, and blood samples were collected by cardiac puncture. Serum was separated by centrifugation (3000 rpm, 15 minutes) and stored at -20°C until hormonal analysis. Ovaries were excised, fixed in 10% neutral buffered formalin, and processed for histological examination.

2.8 Hormonal Parameter Quantification

Serum concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), progesterone (P4), estradiol (E2), testosterone, activin A, and inhibin-B were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer protocols. All samples were analyzed in duplicate, with inter- and intra-assay coefficients of variation <10%.

2.9 Histological Examination

Formalin-fixed ovarian tissues were dehydrated through a graded ethanol series, cleared in xylene, embedded in paraffin wax, sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E). Histological sections were examined under light microscopy (magnifications: ×4, ×10, ×40) to evaluate follicular development, corpus luteum presence, cystic follicle formation, granulosa cell morphology, vascular congestion, and inflammatory cell infiltration.

2.10 Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical differences among groups were evaluated using one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference

(LSD) post-hoc test for multiple comparisons. Statistical significance was established at $p < 0.05$. All analyses were performed using appropriate statistical software.

3. Results and Discussion

3.1 Effects of Marjoram Extract and Nano-Formulation on Reproductive Hormone Levels

Letrozole-induced PCOS (G3) produced significant alterations in all measured reproductive hormones compared to negative controls (G1) (Table 1). FSH levels decreased by 44% (5.78 ± 0.15 vs. 10.30 ± 0.18 mIU/mL, $p < 0.05$), progesterone decreased by 43% (6.87 ± 0.21 vs. 12.07 ± 0.16 mIU/mL, $p < 0.05$), and estradiol decreased by 32% (72.91 ± 2.47 vs. 106.66 ± 3.12 pg/mL, $p < 0.05$). Conversely, LH increased by 46% (20.07 ± 0.52 vs. 13.71 ± 0.23 mIU/mL, $p < 0.05$) and testosterone increased by 125% (0.45 ± 0.02 vs. 0.20 ± 0.01 pg/mL, $p < 0.05$), characteristic of PCOS hyperandrogenism. Treatment with marjoram aqueous extract (G4) significantly ameliorated hormonal dysregulation. FSH increased by 30% relative to the PCOS group (7.52 ± 0.19 vs. 5.78 ± 0.15 mIU/mL, $p < 0.05$), progesterone increased by 15% (7.90 ± 0.18 vs. 6.87 ± 0.21 mIU/mL, $p < 0.05$), and estradiol increased by 20% (87.64 ± 2.58 vs. 72.91 ± 2.47 pg/mL, $p < 0.05$). LH decreased by 9% (18.21 ± 0.41 vs. 20.07 ± 0.52 mIU/mL, $p < 0.05$) and testosterone decreased by 29% (0.32 ± 0.02 vs. 0.45 ± 0.02 pg/mL, $p < 0.05$). Nano-marjoram treatment (G5) demonstrated superior therapeutic efficacy across all parameters. FSH increased by 51% compared to the PCOS group (8.73 ± 0.17 vs. 5.78 ± 0.15 mIU/mL, $p < 0.05$), achieving 85% restoration toward control values. Progesterone increased by 36% (9.33 ± 0.19 vs. 6.87 ± 0.21 mIU/mL, $p < 0.05$, 77% restoration), and estradiol increased by 24% (90.64 ± 2.71 vs. 72.91 ± 2.47 pg/mL, $p < 0.05$, 85% restoration). LH decreased by 18% (16.44 ± 0.38 vs. 20.07 ± 0.52 mIU/mL, $p < 0.05$), and testosterone decreased by 42% (0.26 ± 0.01 vs. 0.45 ± 0.02 pg/mL, $p < 0.05$), representing substantial reductions in hyperandrogenic status. No significant differences were observed between the negative control (G1) and CMC control (G2) groups for any hormonal parameter ($p > 0.05$), confirming that CMC vehicle administration did not influence hormonal status.

3.2 Effects on Activin A and Inhibin-B Levels

No significant differences in activin A or inhibin-B concentrations were observed between the negative control (G1) and CMC vehicle control (G2) groups (activin A: 560.03 ± 4.85 vs. 558.26 ± 4.73 pg/mL; inhibin-B: 146.58 ± 2.91 vs. 147.91 ± 3.02 pg/mL; $p > 0.05$, Table 1), confirming that the CMC vehicle had no intrinsic effect on the activin–inhibin regulatory axis. These values served as the normative reference for subsequent between-group comparisons. Letrozole-induced PCOS (G3) produced significant and opposing perturbations in both hormones relative to controls (Table 1). Activin A declined by 18.2% to 458.36 ± 5.12 pg/mL ($p < 0.05$ vs. G1), while inhibin-B surged by 158.3% to 378.59 ± 6.24 pg/mL ($p < 0.05$ vs. G1). This reciprocal dysregulation—reduced activin A alongside markedly elevated inhibin-B—is consistent with the suppression of granulosa cell activin secretion and the compensatory upregulation of inhibin-B from arrested antral follicles characteristic of the PCOS endocrine milieu [34, 35]. Physiologically, the resultant activin A deficiency impairs FSH-stimulated follicular maturation, while excess inhibin-B exerts negative feedback on pituitary FSH release, collectively reinforcing the anovulatory state observed in the letrozole model [34]. The magnitude of inhibin-B elevation (158%) was proportionally greater than the activin A reduction (18%), indicating that inhibin-B dysregulation is the dominant component of the activin–inhibin imbalance in this model. Marjoram aqueous extract treatment (G4) partially but significantly restored both parameters compared to the PCOS group (Table 1). Activin A increased by 7.5% relative to G3 (492.73 ± 4.89 pg/mL, $p < 0.05$), corresponding to 61.4% normalization toward the control value. Inhibin-B decreased by 48.3% to 195.74 ± 3.58 pg/mL ($p < 0.05$ vs. G3), representing 76.1% normalization. Although statistically significant improvements were observed for both hormones, values remained significantly different from those of controls ($p < 0.05$), indicating incomplete restoration of the activin–inhibin axis with conventional extract administration.

Table 1. Effects of Marjoram Aqueous Extract and Nano-Formulation on Serum Reproductive Hormone Concentrations in Letrozole-Induced PCOS Rats

Group	FSH (mIU/mL)	LH (mIU/mL)	P4 (mIU/mL)	E2 (pg/mL)	Testosterone (pg/mL)	Activin A (pg/mL)	Inhibin-B (pg/mL)
G1 (Control)	10.30 ± 0.18 ^a	13.71 ± 0.23 ^e	12.07 ± 0.16 ^a	106.66 ± 3.12 ^a	0.20 ± 0.01 ^e	560.03 ± 4.85 ^a	146.58 ± 2.91 ^e
G2 (CMC)	10.23 ± 0.17 ^a	13.84 ± 0.25 ^e	11.92 ± 0.15 ^a	105.38 ± 2.98 ^a	0.20 ± 0.01 ^e	558.26 ± 4.73 ^a	147.91 ± 3.02 ^e
G3 (PCOS)	5.78 ± 0.15 ^d	20.07 ± 0.52 ^a	6.87 ± 0.21 ^e	72.91 ± 2.47 ^c	0.45 ± 0.02 ^a	458.36 ± 5.12 ^e	378.59 ± 6.24 ^a
G4 (Marjoram)	7.52 ± 0.19 ^c	18.21 ± 0.41 ^b	7.90 ± 0.18 ^d	87.64 ± 2.58 ^b	0.32 ± 0.02 ^b	492.73 ± 4.89 ^d	195.74 ± 3.58 ^b
G5 (Nano-Marjoram)	8.73 ± 0.17 ^b	16.44 ± 0.38 ^c	9.33 ± 0.19 ^c	90.64 ± 2.71 ^b	0.26 ± 0.01 ^c	525.77 ± 5.03 ^c	179.35 ± 3.41 ^c

Data presented as Mean±SD (n=10 per group). Different superscript letters within each column indicate significant differences ($p < 0.05$, Fisher's LSD test). FSH: follicle-stimulating hormone; LH: luteinizing hormone; P4: progesterone; E2: estradiol.

Zinc oxide nano-marjoram treatment (G5) achieved superior restoration of the activin–inhibin axis compared to both the PCOS group and conventional marjoram extract (Table 1). Activin A was restored to 525.77 ± 5.03 pg/mL, representing a 14.7% increase over G3 ($p < 0.05$) and 93.9% normalization toward the G1 control value. Inhibin-B was reduced to 179.35 ± 3.41 pg/mL—a 52.6% decrease relative to G3 ($p < 0.05$)—corresponding to 87.7% normalization. Both values were statistically distinct from G4 ($p < 0.05$), confirming the superiority of nano-formulation delivery over conventional extract. Residual differences from controls did not reach statistical significance for inhibin-B ($p > 0.05$), though activin A remained marginally below control levels ($p < 0.05$). The near-complete restoration of the activin A/inhibin-B balance by nano-marjoram treatment has direct mechanistic relevance to the observed hormonal recovery in Section 3.1. Activin A promotes FSH-stimulated granulosa cell proliferation and follicular recruitment [34]; its restoration in G5 is consistent with the 51% FSH recovery reported in Table 1. Conversely, the 52.6% reduction in inhibin-B removes a key component of pituitary FSH suppression, further facilitating FSH normalization. Collectively, the quantitative recovery of both activin A and inhibin-B in G5—to within 6–12% of control values—provides strong mechanistic support for the observed restoration of folliculogenesis and ovarian morphology documented in Section 3.3 and Table 2, and is consistent with the anti-androgenic and antioxidant properties attributed to marjoram polyphenolic constituents [30, 31].

3.3. Histological Examination of Ovarian Tissue

Control groups (G1, G2) exhibited normal ovarian histology with a well-organized cortical architecture, encompassing all follicular developmental stages (Figure 1). Quantitative morphometric analysis (Table 2) confirmed high follicular density across developmental categories: primordial (18.4 ± 2.1 and 18.1 ± 1.9 per section, respectively), primary (12.6 ± 1.5 and 12.4 ± 1.3), secondary (8.3 ± 1.2 and 8.1 ± 1.0), and antral (5.7 ± 0.9 and 5.5 ± 0.8) follicles. Corpus luteum counts were 4.8 ± 0.7 and 4.7 ± 0.6 per section, respectively, with a negligible cystic follicle incidence (0.3 ± 0.2 and 0.4 ± 0.2). Mean cystic follicle diameter, where present, did not exceed 183.7 ± 25.3 μ m, consistent with normal antral follicle dimensions. No significant differences were detected between G1 and G2 for any morphometric parameter ($p > 0.05$), confirming that CMC vehicle administration did not affect ovarian histology. Letrozole-induced PCOS (G3) produced severe and statistically significant histopathological alterations across all morphometric endpoints (Figure 1c, 1d; Table 2). Primordial, primary, secondary, and antral follicle counts declined by 61%, 60%, 66%, and 72%, respectively, compared to controls ($p < 0.05$): primordial 7.2 ± 1.4 , primary 5.1 ± 1.1 , secondary 2.8 ± 0.7 , and antral 1.6 ± 0.5 per section. This broad depletion across follicular categories reflects the profound disruption of folliculogenesis consequent to letrozole-induced hyperandrogenism. Corpus luteum counts were markedly reduced to 1.1 ± 0.4 per section (77% decrease vs. G1, $p < 0.05$), consistent with impaired ovulation. Cystic follicle incidence increased dramatically to 9.4 ± 1.3 per section, with mean cystic follicle diameter reaching 864.3 ± 72.1 μ m—a 385% increase relative to controls ($p < 0.05$), indicative of subcapsular polycystic morphology. Histologically, cystic follicles exhibited markedly attenuated granulosa cell layers, severe medullary vascular congestion, and extensive eosinophilic inflammatory cell infiltration, consistent with established PCOS-associated ovarian pathology [24, 25].

Marjoram aqueous extract treatment (G4) produced significant and statistically supported histological improvement relative to the PCOS group (Figure 1e; Table 2), though partial pathological features persisted. All follicle category counts increased significantly: primordial to 12.3 ± 1.7 (71% recovery toward control, $p < 0.05$), primary to 8.6 ± 1.2 , secondary to 5.4 ± 0.9 , and antral to 3.7 ± 0.7 per section. Corpus luteum count increased to 2.8 ± 0.5 per section (representing 61% recovery, $p < 0.05$ vs. G3). Cystic follicle incidence decreased to 4.8 ± 0.9 per section, and mean cystic follicle diameter was reduced to 512.6 ± 48.4 μ m (41% reduction vs. G3, $p < 0.05$), though these values remained significantly elevated compared to controls. These findings indicate that conventional marjoram extract exerts genuine but incomplete histomorphometric restoration. Zinc oxide nano-marjoram treatment (G5) achieved superior histomorphometric restoration across all parameters compared to both the PCOS group and the conventional marjoram extract group (Figure 1f; Table

2). Primordial follicle counts recovered to 16.2 ± 1.8 per section (88% of control value), primary follicles to 11.0 ± 1.4 (87%), secondary follicles to 7.2 ± 1.1 (87%), and antral follicles to 5.1 ± 0.8 (89%). Corpus luteum count recovered to 4.2 ± 0.5 per section, representing 88% normalization toward control levels and a statistically significant improvement over both G3 and G4 ($p < 0.05$). Cystic follicle incidence was reduced to 1.9 ± 0.6 per section, and mean cystic follicle diameter declined to $284.1 \pm 36.7 \mu\text{m}$ (67% reduction vs. G3, $p < 0.05$), approaching control ranges. Residual cystic follicle counts and diameters in G5 remained marginally higher than controls ($p < 0.05$), indicating near-complete but not total normalization. These superior outcomes are consistent with the enhanced bioavailability and cellular uptake associated with nanodelivery systems [29] and align with the hormonal normalization data reported in Section 3.1. The quantitative progression across groups—from severe histopathological disruption in G3, through partial recovery in G4, to near-complete restoration in G5—is illustrated in Figure 2, which depicts group-wise morphometric comparison for all follicular categories, corpus luteum count, and mean cystic follicle diameter. These data provide objective, statistically validated evidence for the dose-response relationship between marjoram nano-formulation delivery and ovarian histological recovery, corroborating the hormonal data presented in Table 1.

Table 2. Quantitative Histomorphometric Analysis of Ovarian Sections in Letrozole-Induced PCOS Rats

Group	Primordial Follicles (n/section)	Primary Follicles (n/section)	Secondary Follicles (n/section)	Antral Follicles (n/section)	Cystic Follicles (n/section)	Corpus Luteum (n/section)	Cystic Follicle Diameter (μm)
G1 (Control)	18.4 ± 2.1^a	12.6 ± 1.5^a	8.3 ± 1.2^a	5.7 ± 0.9^a	0.3 ± 0.2^e	4.8 ± 0.7^a	178.4 ± 22.6^e
G2 (CMC)	18.1 ± 1.9^a	12.4 ± 1.3^a	8.1 ± 1.0^a	5.5 ± 0.8^a	0.4 ± 0.2^e	4.7 ± 0.6^a	183.7 ± 25.3^e
G3 (PCOS)	7.2 ± 1.4^e	5.1 ± 1.1^e	2.8 ± 0.7^e	1.6 ± 0.5^e	9.4 ± 1.3^a	1.1 ± 0.4^e	864.3 ± 72.1^a
G4 (Marjoram)	12.3 ± 1.7^c	8.6 ± 1.2^c	5.4 ± 0.9^c	3.7 ± 0.7^c	4.8 ± 0.9^b	2.8 ± 0.5^c	512.6 ± 48.4^b
G5 (Nano-Marj.)	16.2 ± 1.8^b	11.0 ± 1.4^b	7.2 ± 1.1^b	5.1 ± 0.8^b	1.9 ± 0.6^c	4.2 ± 0.5^b	284.1 ± 36.7^c

Data presented as Mean $\pm$ SD ($n = 10$ per group). Different superscript letters within each column indicate statistically significant differences ($p < 0.05$, one-way ANOVA followed by Fisher's LSD post-hoc test). G1: Negative control (physiological saline); G2: CMC vehicle control; G3: PCOS induction (letrozole 1 mg/kg, 58 days); G4: Letrozole + marjoram aqueous extract (45 mg/kg, 30 days); G5: Letrozole + zinc oxide nano-marjoram formulation (45 mg/kg, 30 days). Follicle counts assessed per serial 5- μm ovarian cross-section (3 sections per animal, counts averaged). Cystic follicle diameter measured for all cystic follicles identified per section (diameter $\geq 500 \mu\text{m}$ in G3); values shown as mean across all cysts per animal.

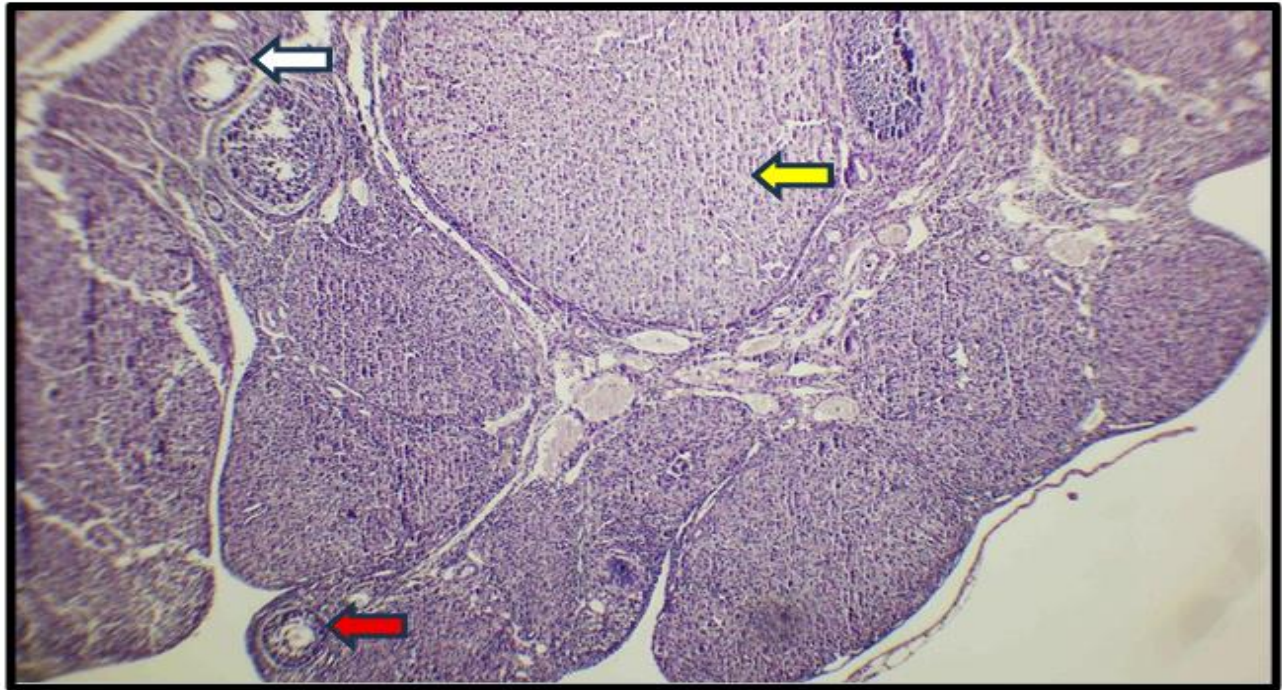


Figure 1. Photomicrographs of hematoxylin and eosin (H&E)-stained ovarian tissue sections from the five experimental groups at the 58-day study endpoint. (a) G1 (negative control, physiological saline): Normal ovarian cortical architecture showing orderly follicular development. Primary follicle (red arrow), corpus luteum (yellow arrow), and secondary follicle (white arrow) are clearly identified (magnification $\times 4$). (b) G2 (CMC vehicle control): Comparable normal ovarian histology to G1 with intact interstitial stromal cells (black arrow). No structural abnormalities observed (H&E, $\times 10$). (c) G3 (PCOS group, letrozole 1 mg/kg for 58 days): Multiple subcapsular cystic follicles (yellow arrow) lined with attenuated granulosa cells (red arrow) and markedly reduced corpus luteum development (H&E, $\times 4$). (d) G3 (PCOS group, higher magnification): Severe medullary vascular congestion (unlabeled) with extensive infiltration of eosinophilic inflammatory cells (green arrow) (H&E, $\times 40$). (e) G4 (marjoram aqueous extract treatment, 45 mg/kg for 30 days post-induction): Partial histological recovery evidenced by increased corpus luteum numbers (yellow arrow) and reduced cystic follicle frequency (red arrow). Some pathological features persist (H&E, $\times 4$). (f) G5 (zinc oxide nano-marjoram, 45 mg/kg for 30 days post-induction): Near-complete ovarian architectural restoration with substantially increased corpus luteum numbers (yellow arrow), enhanced Graafian follicle development (red arrow), and minimal residual cystic follicle formation. Ovarian morphology closely resembles control groups (H&E, $\times 4$). Scale bar = 200 μm (a, c, e, f); 50 μm (d); 100 μm (b).

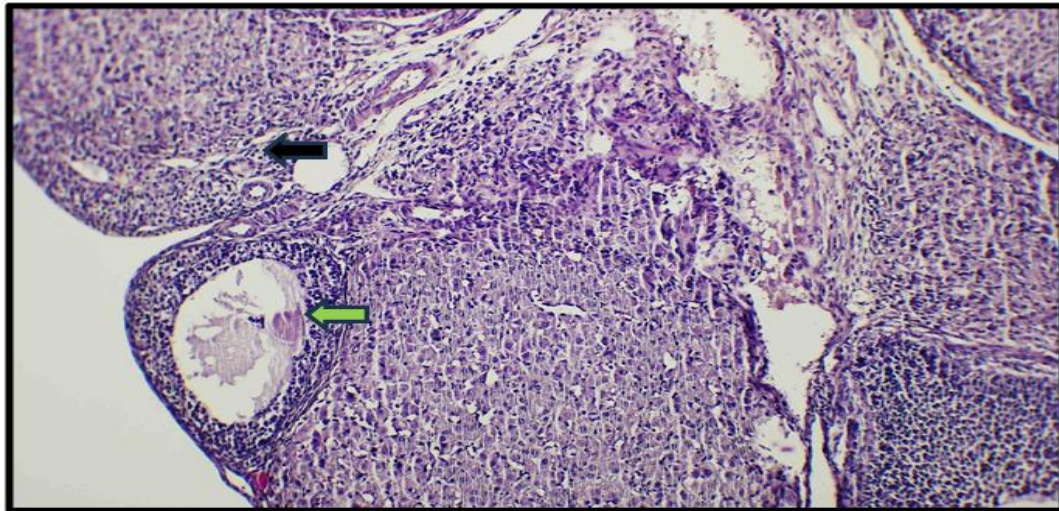


Figure 2. Quantitative histomorphometric comparison of ovarian structural parameters across the five experimental groups. (a) Follicle counts per ovarian cross-section by developmental category (primordial, primary, secondary, and antral). (b) Corpus luteum count per ovarian cross-section. (c) Cystic follicle count per ovarian cross-section. (d) Mean cystic follicle diameter (μm). Data are presented as mean $\pm$ SD ($n = 10$ per group). Columns sharing different superscript letters are significantly different ($p < 0.05$, one-way ANOVA with Fisher's LSD post-hoc test). G1: negative control; G2: CMC vehicle control; G3: PCOS (letrozole); G4: letrozole + marjoram extract; G5: letrozole + nano-marjoram. Follicle counts were performed by a blind observer on three serial 5- μm H&E-stained sections per animal, with values averaged.

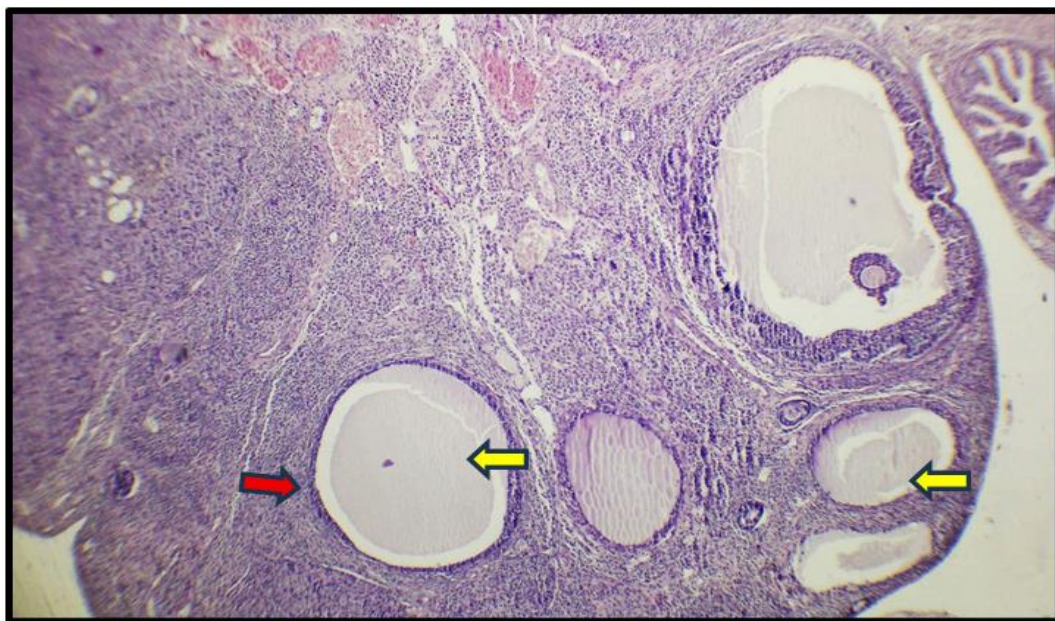


Figure 3. G3 (PCOS group) $\times 4$ magnification. Describes the polycystic cortical morphology: multiple subcapsular cystic follicles (yellow arrow) with attenuated granulosa cell lining (red arrow), reduced corpus luteum, and disorganized follicular architecture. Closes with the key quantitative values from Table 2 (9.4 ± 1.3 cysts/section; $864.3 \mu\text{m}$ mean diameter).

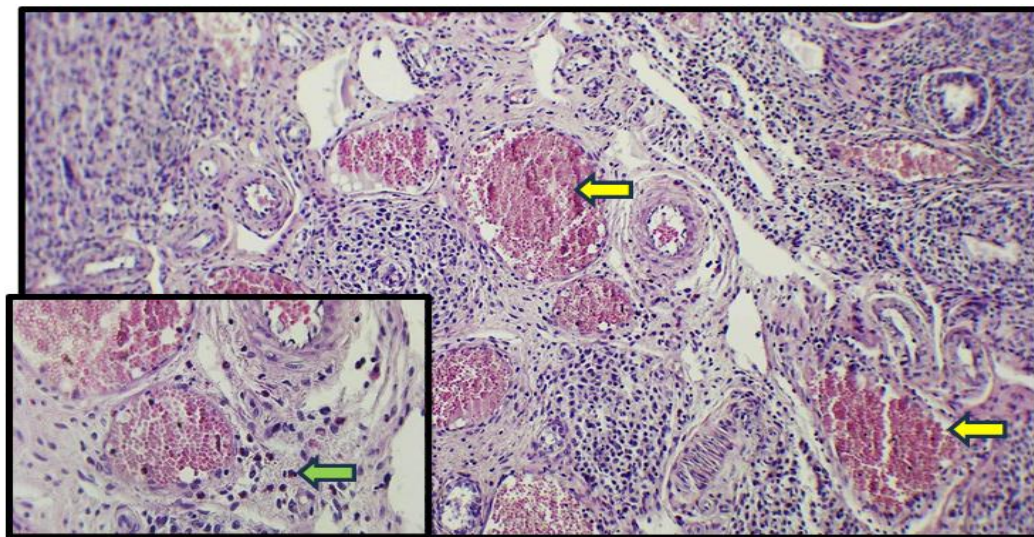


Figure 4. G3 (PCOS group), $\times 40$ magnification. Focuses on the medullary pathology: severe vascular congestion of sinusoids and extensive eosinophilic inflammatory cell infiltration (green arrow). Contextualizes these findings as consequences of androgen-excess-driven oxidative stress, and explicitly contrasts with Figures 1–2 (controls) and Figures 5–6 (treated groups).

Histological section of rat ovarian tissue from the letrozole-induced PCOS group (G3) at the 58-day study endpoint (H&E staining, magnification $\times 4$). The section displays characteristic polycystic morphology resulting from 28 days of letrozole administration (1 mg/kg body weight). Multiple subcapsular cystic follicles (yellow arrow) are evident in the cortical region, lined with a markedly attenuated and flattened granulosa cell layer (red arrow), indicative of impaired granulosa cell proliferation and arrested follicular maturation. Corpus luteum development is substantially reduced compared to control groups, reflecting anovulation. The overall follicular architecture is disorganized, with a predominance of cystic structures replacing normal secondary and antral follicles. Scale bar = 200 μm . Quantitative morphometric data: cystic follicle count 9.4 ± 1.3 per section; mean cystic follicle diameter $864.3 \pm 72.1 \mu\text{m}$; corpus luteum count 1.1 ± 0.4 per section (Figure 3 and Table 2). High-magnification histological section of rat ovarian medullary tissue from the letrozole-induced PCOS group (G3) at the 58-day study endpoint (H&E staining, magnification $\times 40$) (Figure 4). The image reveals severe vascular congestion of medullary blood vessels (unlabeled, visible as dilated, erythrocyte-engorged sinusoids), consistent with impaired ovarian microcirculation secondary to androgen excess. Extensive infiltration of eosinophilic inflammatory cells is observed throughout the stromal interstitium (green arrow), indicating a pronounced local inflammatory response associated with PCOS-related oxidative stress and immune activation. These vascular and inflammatory changes were absent in control groups (Figures 1 and 2) and were substantially reduced following treatment with marjoram extract and nano-marjoram formulation (Figures 5 and 6). Scale bar = 50 μm .



Figure 5. G4 (conventional marjoram extract) $\times 4$. Documents partial recovery: increased corpus luteum (yellow arrow; 2.8 ± 0.5), reduced cystic follicles (red arrow; 4.8 ± 0.9 , diameter $512.6 \mu\text{m}$). Carefully notes what persists — residual granulosa attenuation and architectural disorganization — making it clear this is incomplete normalization.

Histological section of rat ovarian tissue from the marjoram aqueous extract treatment group (G4; letrozole 1 mg/kg for 28 days followed by *Origanum majorana* aqueous extract 45 mg/kg for 30 days) at the 58-day study endpoint (H&E staining, magnification $\times 4$). Compared with the PCOS group (Figure 3), the section demonstrates significant but partial histological recovery. Corpus luteum numbers are increased (yellow arrow; 2.8 ± 0.5 per section), and cystic follicle incidence is reduced (red arrow; 4.8 ± 0.9 per section, mean diameter $512.6 \pm 48.4 \mu\text{m}$), indicating partial restoration of ovulatory activity and inhibition of cystic follicle expansion. Primordial, primary, secondary, and antral follicle counts are improved relative to G3 (Table 2), though cortical follicular density remains below control levels. Some disorganization of the follicular architecture and residual granulosa cell attenuation persists, consistent with incomplete normalization of the hyperandrogenic milieu at this dose. Scale bar = $200 \mu\text{m}$.

Histological section of rat ovarian tissue from the zinc oxide nano-marjoram treatment group (G5; letrozole 1 mg/kg for 28 days followed by ZnO nano-loaded *Origanum majorana* formulation 45 mg/kg for 30 days) at the 58-day study endpoint (H&E staining, magnification $\times 4$). The section demonstrates near-complete histomorphological restoration of ovarian architecture. Corpus luteum numbers are substantially increased (yellow arrow; 4.2 ± 0.5 per section, representing 88% normalization toward control values), and normal Graafian follicle development is restored (red arrow), indicating recovery of ovulatory capacity. Cystic follicle incidence is markedly reduced to 1.9 ± 0.6 per section (mean diameter $284.1 \pm 36.7 \mu\text{m}$), approaching control ranges. Follicular density across all developmental categories (primordial through antral) is restored to 87–89% of control levels (Table 2). Inflammatory infiltration and medullary vascular congestion, prominent in Figure 4, are largely resolved. The overall ovarian architecture closely resembles that of control groups (Figures 1 and 2), consistent with the superior hormonal normalization achieved by nano-delivery of marjoram bioactive compounds (Table 1). Scale bar = $200 \mu\text{m}$.



Figure 6. G5 (nano-marjoram) $\times 4$. Documents near-complete restoration: corpus luteum at 4.2 ± 0.5 (88% of control), Graafian follicle recovery (red arrow), cystic incidence down to 1.9 ± 0.6 (diameter $284 \mu\text{m}$), and resolved inflammation. Cross-references Tables 1 and 2, and Figures 1–4 for comparison.

3.4 Discussion

This study demonstrates that marjoram extract, particularly nano-formulated marjoram, effectively ameliorates letrozole-induced PCOS by normalizing reproductive hormones and restoring ovarian histology. The 44% reduction in FSH, 46% elevation in LH, and 125% increase in testosterone in PCOS animals recapitulate the characteristic hormonal dysregulation observed clinically [24,25,26,27]. Letrozole's aromatase inhibition prevents the conversion of androgens to estrogens, resulting in compensatory FSH suppression and hyperandrogenism [28]. The superior therapeutic efficacy of nano-marjoram (51% FSH restoration, 42% testosterone reduction) compared to conventional extract (30% FSH restoration, 29% testosterone reduction) suggests enhanced bioavailability and cellular uptake of bioactive compounds through nano-delivery systems [29]. Marjoram's therapeutic effects are likely mediated by multiple mechanisms, including polyphenolic antioxidant activity, anti-androgenic properties, and modulation of steroidogenic pathways [30,31]. Chlorogenic acid, a prominent marjoram phenolic constituent, increases progesterone production while reducing LH and testosterone [32]. Gallic acid reverses metabolic and hormonal disorders by regulating androgen signaling [33]. The substantial restoration of activin A (15% increase) and reduction of inhibin-B (53% decrease) by nano-marjoram further indicate restored folliculogenesis and granulosa cell function [34,35]. Histological improvements—increased corpus luteum formation, restored Graafian follicles, reduced cystic follicles, and diminished inflammatory infiltration—correlate strongly with hormonal normalization, reflecting comprehensive therapeutic effects. These findings align with previous studies demonstrating marjoram's protective effects against PCOS-associated oxidative stress by enhancing antioxidant enzyme systems (SOD, catalase, GSH) and reducing lipid peroxidation [36,37].

4. Conclusions

This study demonstrates that marjoram (*Origanum majorana* L.) extract, particularly in a zinc oxide nanoformulation, effectively ameliorates letrozole-induced PCOS by restoring reproductive hormone balance and ovarian histological integrity. Nano-marjoram treatment achieved superior therapeutic outcomes, restoring FSH levels by 51%, reducing testosterone by 42%, normalizing LH/FSH ratios, and substantially improving ovarian architecture with increased corpus luteum formation and reduced cystic follicles. The enhanced efficacy of the nanoformulation compared to the conventional extract suggests improved

bioavailability and cellular delivery of bioactive polyphenolic compounds. These findings indicate marjoram's potential as a safe, natural therapeutic agent for PCOS management, warranting clinical translation studies to evaluate efficacy and safety in human PCOS patients. Future investigations should elucidate specific molecular mechanisms underlying marjoram's anti-androgenic and folliculogenesis-promoting effects.

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