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Research Article

Aqueous Ethanolic Extract of *Inonotus obliquus* Ameliorates Polycystic Ovarian Syndrome by Modulating Oxidative Stress and Downregulating TNF-α and Interleukin-6

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Polycystic ovarian syndrome (PCOS) is a complex metabolic and endocrine disorder with multifactorial etiology and complex pathogenesis. These intrinsic physiological changes cause anovulation, infertility, and miscarriage in women and exacerbate their chances of becoming hyperlipidemic and diabetic. *Inonotus obliquus* has been used traditionally for infertility problems. The present study aimed to investigate the therapeutic effects of aqueous ethanolic extract of *Inonotus obliquus* (AEIO) in female rats with letrozole-induced PCOS, along with the determination of its possible mechanism. HPLC analysis revealed the presence of gallic acid, chlorogenic acid, rutin, *p*-coumaric, benzoic acid, quercetin, salicylic acid, and kaempferol. Thirty female albino rats were acquired and divided into two groups (5 + 25) to induce PCOS. Letrozole (1 mg/kg) was used to induce the disease for 7 weeks (25 rats) except for the normal control (5 rats). The disease was confirmed by vaginal smear cytology, weight gain, and endocrinopathy. After disease induction, rats were divided into five groups (five rats in each group; disease control, metformin 20 mg/kg, AEIO 100, 300, and 500 mg/kg). After completion of the study, the animals were euthanized under the influence of anesthesia (chloroform). Ovaries were removed for histopathology, the liver was evaluated for oxidative stress biomarkers, and blood samples were collected for biochemical evaluation. Ovarian histopathology showed an abnormal architecture with cystic follicles and abnormal granulosa cells. Interestingly, treatment with AEIO restored normal ovarian histology with primary, growing, and developing follicles. After conducting a hormonal analysis, it was found that the induction of PCOS led to a significant increase ($p < 0.001$) in luteinizing hormone (LH), insulin, and testosterone levels, while the levels of follicle-stimulating hormone (FSH) decreased. However, treatment with AEIO at doses of 100–500 mg/kg restored these levels to normal. PCOS induction also resulted in oxidative stress and lipid peroxidation by significantly decreasing antioxidant enzymatic markers (SOD, CAT, and GSH) and increasing levels of lipid peroxidation enzymatic markers (MDA). AEIO restored the levels of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), and reduced the level of malondialdehyde (MDA). In conclusion, antioxidant phytochemicals (gallic acid, chlorogenic acid, rutin, *p*-coumaric, benzoic acid, quercetin, salicylic acid, and kaempferol) rich extract alleviated PCOS symptomatology through modulating oxidative stress markers and eliminating ovarian low-grade inflammation by downregulating the expression of NF-κB associated TNF-α and IL-6.

1. Introduction

Polycystic ovarian syndrome (PCOS) is a multifaceted spectrum of endocrinopathies and is the most common cause of infertility. The onset point of this disease is ambiguous. The total percentage of females suffering from PCOS is approximately 5-20% [1]. The persistent anomalous condition of hormones leads to morphological changes in the ovaries (cyst/fluid-filled sac formation). The benchmark criteria (Rotterdam) for PCOS are the presence of hyperandrogenism, cysts (at least 12), and anovulation or at least 2 of these factors must be present. An uncontrolled increase in the number of cystic follicles occurs along with growth retardation [2, 3]. The signs and symptoms of PCOS are acne, dysregulated menstruation (once every 3 or 6 months), miscarriage, stubborn lower body fat, and excessive male-like hair patterns on the face and body. Self-assessment of these symptoms could help in the early diagnosis of PCOS [1]. PCOS is divided into four types depending on the level of severity of the disease. The phenotype with insulin resistance and hyperandrogenism, along with signs and symptoms, is considered a severe type of PCOS [4]. Comorbidities that further enhance the vulnerability of PCOS include epilepsy, depression, psychosis, and bipolar disorder [5]. The alterations presented by PCOS could be due to genetic defects or environmental factors. The offspring of PCOS patients are more likely to suffer from the same condition. Genetic mutations change endocrine and metabolic functions [2]. Persistence in physical inactivity is another key factor, as it induces metabolic disturbance and obesity. Most of the PCOS patients are obese along with insulin resistance. For the sake of management, lifestyle modifications, diet modification (healthy and organic), and exercise (weight management) are of utmost importance due to the ambiguity in finding the actual cause and the beginning of disease [2].

The hormonal imbalance could be due to dysregulation of the concentration, clearance, sensitivity, and activity of hormonal receptors. All of these predicaments ultimately cause cyst formation [6]. Abnormal functioning of the hypothalamus, adrenal gland, pituitary gland, granulosa, and theca cells causes aberrations in vital hormones including LH, FSH, AMH, GnRH, leptin, androgens, insulin, and their receptors. This malfunctioning causes follicular arrest [7, 8]. The lower availability of dopaminergic receptors alters the reward system and causes obesity [9]. The hormones with a high prognostic value for PCOS are testosterone, FSH, LH, and insulin, while adiponectin, irisin, and inhibin have less prognostic value [10]. Hyperandrogenemia is one of the key features in the pathogenesis of PCOS. The ovarian cells keep synthesizing androgens which induce high insulin levels and the development of resistance in the body. The negative effects exerted by excessive and persistent androgen levels in the body include the short response of the FSH receptor to its hormone, inadequate secretion of progesterone and estrogen, retardation of follicle maturation, and finally causes problems in pregnancy. FSH plays a crucial role in ovulation; when its level is disturbed, the regular cycle is disturbed. Selection and maturation of a particular follicle for ovulation doesn't occur [11]. PCOS patients exhibit elevated proinsulin and insulin in the body. The clearance of these hormones is also reduced. Both obese and nonobese PCOS patients have increased oxidative stress due to high circulating sugar levels. Obesity and insulin resistance disturb various glands and organs and cause anovulation, delayed periods, elevated androgens, LH, and reduced leptin levels. Both reduced and elevated leptin levels prevent or even terminate pregnancy and lead to reproductive dysfunction [7, 12].

Estrogen stimulates leptin release. According to studies, signaling via gonadotrophin-releasing hormone (GnRH) regulates the synthesis and release of LH. Aberrations in GABA and leptin activity affect the release of GnRH. Hyperactivated GABA neurons and reduced leptin levels reduce GnRH activity that ultimately disturbs the level of LH and progesterone [2, 5]. Sex hormone-binding globulin (SHBG) regulates the bioavailability of sex hormones and their removal from the body. Its concentration is below normal in PCOS, which causes inflammation, obesity, and metabolic syndrome [13]. Trends in maintaining health and fitness are tremendously increasing, and people are more focused on using pure, organic foods and medicines. The main reason behind this is that organic medicine provides long-lasting health benefits with little or no side effects. Medicinal plant-based herbal medicines and functional food have gained a lot of attention in recent times due to the presence of natural antioxidants [14–16]. Modern medicine comes with a lot of adverse effects. Researchers are putting more effort into studying the therapeutic effects of plants and their phytochemicals in the treatment of various diseases. Studies have shown that *Inonotus obliquus* (IO) has improved insulin sensitization, increased kidney functions that were disturbed due to diabetes, reduced the level of free radicals, and reduced blood glucose levels in the streptozotocin-induced diabetic animal model. This hypoglycemic effect is particularly obtained by the polysaccharides of *Inonotus obliquus* [17, 18].

Inonotus obliquus, also known as the Chaga mushroom, is commonly found in China, Russia, Korea, Canada, Siberia, Europe, Asia, and other countries. It belongs to the *Hymenochaetacea*e family and usually grows on old trees [19]. It can grow up to 40 cm in length [20]. *Inonotus obliquus* has several triterpenoids with potential pharmacological activities. Two particular lanostanes present in *Inonotus obliquus* possess anti-inflammatory activity [21]. The phenolic compounds present in *Inonotus obliquus* have potential antioxidant properties. Chaga has strong radical scavenging properties due to the vital phytochemicals it contains. In addition to the phytochemicals mentioned above, melanin also plays a role in scavenging free radicals [22]. Other phytochemical constituents include triterpenoids, betulin, betulinic acid, inositol, gallic acid, polysaccharides, sterols, polyphenols, lupeol, melanin, and ergosterol [19, 21, 23]. People have been using Chaga for the treatment of various diseases, but its uses are neither well-studied nor well-documented. Before this study, its effects were neither determined nor documented in PCOS.

2. Materials and Methods

2.1. Apparatus and Equipment. The equipment utilized during the entire study included sterile beakers, a stirrer, tests tubes, falcon tubes, a Petri dish, a magnetic stirrer, a digital pH meter, falcon tubes, Eppendorf tubes, micropipette, weighing balance, Whatman filter paper, sonicator, UV-visible spectrophotometer, pestle, and mortar.

2.2. Extract Acquisition. The aqueous ethanolic extract of *Inonotus obliquus* (AEIO) in fine powdered form was obtained from Sigma Algronic, a Karachi-based extract supplier company. This extract was used in rats induced by PCOS to evaluate its effectiveness in PCOS.

2.3. In vitro Antioxidant Testing. Free radicals cause cell death by inducing a highly stressful environment within the cell. Some molecules prevent this oxidization by binding to free radicals. This effect is termed antiradical activity. Some molecules (antioxidants) act by stopping the oxidative stress pathway. The antioxidants have been classified based on their functionality or their origin (endogenous or exogenous) for providing the required effect. This activity can be performed using HPLC, DPPH, TPC, and TFC [24].

2.3.1. Evaluation of Radical Scavenging Activity. The DPPH (α, α-Diphenyl-β-Picrylhydrazyl) method (Diphenyl-Picrylhydrazyl) was first introduced in the twentieth century by Marsden S. Blois. It is a stable, free radical that is used to assess the antioxidant capacity of substances. If the subject of the study has anti-oxidant potential, it will reduce the free radical by donating its hydrogen atom [24].

The chemicals used for this test are methanol, DPPH, and AEIO extract. Ethanol was used to make solutions with different concentrations. The 0.1 mM DPPH solution was made in methanol. Ascorbic acid was used as the standard. A 1.6 mL extract solution of different concentrations was taken in a sterile test tube, followed by the addition of 2.4 mL of DPPH solution. After quickly shaking the solution, it was allowed to rest for about half an hour in the dark and then the absorbance was taken at 517 nm in the spectrophotometer [24].

The activity was measured using the following formula.

$$\%\text{DPPH Radical Scavenging Activity} = \frac{A0 - A1}{A0} \times 100. \quad (1)$$

Here, $A0$ is the absorbance of the control, and $A1$ is the absorbance of the extract.

2.3.2. Assessment of Total Phenolic Content (TPC). The TPC of the AEIO was determined using the Chang et al. protocol with the minimal changes studied in another phytochemical analysis study [25]. The sterile test tubes were utilized and gallic acid was used as standard. The sample solution was prepared as 1 mg of AEIO in 1 mL of distilled water. The 0.2 mL sample solution was taken in the test tube, followed by the addition of 0.2 mL standard and 0.2 mL "Folin–Ciocalteu" reagent. The mixture was shaken and left for 4 minutes. 15% sodium carbonate solution was prepared, and 1 mL of sodium carbonate solution was added and kept at room temperature for about 2 hours. Then, the absorbance was recorded at 760 nm on the spectrophotometer. The whole procedure was repeated three times. The whole TPC content was evaluated via the following equation:

$$\text{TPC} = \frac{\textbf{Gallic}\ \text{acid equivalents}\ (\textbf{µg}) \times \textbf{extract}\ \text{volume}}{\textbf{Sample}\ (\textbf{grams})}. \quad (2)$$

2.3.3. Assessment of Total Flavonoid Content (TFC). The TFC of the IO extract was determined using the Chang et al. protocol with minute changes studied in another phytochemical analysis study [25]. Quercetin was used as standard. The chemicals used were aluminum nitrate (10%) and potassium acetate solution (1 M). The 0.2 mL sample solution was taken in the test tube, and the 0.2 mL standard was taken in another test tube. 0.1 mL of Al (NO_3) 3 and 0.1 mL of CH_3CO_2K were added to the test tubes followed by the addition of 4 mL of distilled water. The mixture was incubated at room temperature for about 45 minutes. Then, it was run on the spectrophotometer at 415 nm to check the absorbance. This entire procedure is repeated three times. The complete TFC content was then evaluated using the following formula:

$$\text{TFC} = \frac{\textbf{Quercetin}\ \text{equivalents}\ (\textbf{µg}) \times \textbf{extract}\ \text{volume}}{\textbf{Sample}\ (\textbf{grams})}. \quad (3)$$

2.4. Estimation of Oxidative Stress Biomarkers. The liver is a vital organ that performs tremendous processes like detoxification, maintaining an oxidative stress-free environment in the body. The liver of the rats was used to evaluate the antioxidant activity. Oxidation could be due to the oxidative radical count that becomes too high to destroy the cells or the drug/medication taken exerts a negative impact on these enzymes. So, it is very important to evaluate the activity of these enzymes (SOD, CAT, and GSH) and MDA (lipid peroxidation end-product) [26]. The livers were cleaned with normal saline and dipped in a 20% formalin solution, which was made by mixing 20 mL of formalin in 80 mL of distilled water for organ fixation.

2.4.1. Determination of Superoxidase Dismutase (SOD) Level. Chemicals used for the evaluation of SOD activity are 1.2 mL of phosphate buffer solution (PBS), with an 8.3 pH, 100 μL of liver homogenate, 300 μL nitro blue tetrazolium, 200 μL triton X, 7% glacial acetic acid (to end the reaction), 4 mL of n-butanol, and 100 μL phenazine methosulfate. The homogenate was taken in a sterile test tube and PBS was added to it, followed by phenazine methosulfate. Then, nitroblue tetrazolium and triton X were added to it. The mixture was incubated for 1.5 minutes at room temperature. After the

addition of glacial acetic acid, n-butanol was added, and the test tube was shaken vigorously. After ten minutes of break, the mixture was subjected to centrifugation at 1,000 rotations per minute. The supernatant was separated in a separate tube. Then, the absorbance was taken at 560 nm. Readings were recorded and compared with the standard. N-butanol was used as a blank in the test [27].

2.4.2. Determination of Catalase (CAT) Level. The chemicals used for the analysis of CAT are PBS solution, liver homogenate supernatant, distilled water (DW), and 30 mM hydrogen peroxide (H_2O_2). The H_2O_2 solution was prepared by mixing 0.05 mL of hydrogen peroxide in 50 mL of DW. This test was carried out by taking 0.1 mL of liver homogenate in a test tube. PBS (phosphate buffer solution) of 1.95 mL was added to it. Then, 1 mL of H_2O_2 was in it. The spectrophotometer reading was set at 240 nm and then the samples were run one by one [28]. The recorded activity was then measured using the following formula:

$$\text{CAT} = \frac{\boldsymbol{\delta OD}}{\mathbf{E} \times \mathbf{V} \times \mathbf{mg}\,\text{of protein}}. \quad (4)$$

Here, δOD represented change in the absorbance per minute. V represented volume of tested Sample, E represented Hydrogen Peroxide Extinction Coefficient (0.071mmol cm^{-1}).

2.4.3. Determination of Malondialdehyde (MDA) Level. The chemicals used for this test are a tissue homogenate supernatant, a 0.38% thiobarbituric acid solution (TBA), a 0.25 M HCl solution, and a 15% trichloroacetic acid (TCA) solution. The TBA solution was prepared by mixing 0.38 g of TBA in 100 mL of distilled water. The 15% TCA solution was prepared by mixing 15 g of TCA in 100 mL of distilled water. The 0.25 M hydrochloric acid solution was prepared by mixing 2.5 mL of HCl in 22.5 mL of distilled water. 1 mL of homogenate was taken in a sterile test tube. Then, 3 mL of TBA solution and 2.5 mL of HCl solution were added to it, followed by the addition of TCA solution. After mixing, it was incubated for about 15 minutes. After it was cooled, it was centrifuged again for 10 minutes at 3,500 revolutions per minute. The reading of the spectrophotometer was set at 532 nm and then the samples were run one by one to analyze and record their absorbance. The whole procedure was repeated three times [28]. The recorded activity was then measured as nanomole per mg of tissue protein using the following formula:

$$\text{MDA} = \frac{\mathbf{Y} \times \mathbf{100} \times \mathbf{Vt}}{\mathbf{1.56} \times \mathbf{10^5} \times \mathbf{Wt} \times \mathbf{Vu}}. \quad (5)$$

Here, total Vt total volume of assay mixture (4 mL), Vu Volume of Aliquots, *Y* absorption, Wt weight of the organ (g), 1.56 10^5 molar extinction coefficients.

2.4.4. Determination of Glutathione (GSH) Level. The chemicals for this test, other than tissue homogenate, are 10% TCA, buffer solution, 5,5-dithiobis-2-nitrobenzoic acid (DTNB) reagent, and methanol. The 10% TCA solution was made by dissolving 10 g of TCA in 90 mL of distilled water. The DTNB solution was made by dissolving 29.87 mg of DNTB in 25 mL of methanol. One milliliter of homogenate was taken in a sterile test tube followed by the addition of 1 mL of TCA solution, 4 mL of buffer solution, and 0.5 mL of DTNB. Subsequently, the mixture was run on the spectrophotometer and the absorbance was taken at 412 nm [28]. The GSH activity was calculated using the following formula:

$$\text{GSH} = \frac{\mathbf{Y - 0.00314}}{\mathbf{0.0314}} \times \frac{\mathbf{DF}}{\mathbf{T} \times \mathbf{Vu}}. \quad (6)$$

Here, *Y* absorption, DF Dilution Factor (1), Vu Volume of Aliquots, *T* Tissue homogenate.

2.5. In Vivo Studies

2.5.1. Handling of Animals. Thirty healthy female albino rats (150–200 g) were procured and kept in stainless steel cages at the Animal House of the Faculty of Pharmaceutical Sciences, GCUF. Handling and housing conditions were followed according to protocol. The animal cages were smooth and clean, with enough space for the animals to easily move around. The temperatures were kept between $22 \pm 3^{\circ}$C because its maintenance plays an important role in the regulation of behavior, as well as metabolic pathways. The rats were housed for the first 5–7 days to make them friendly to the environment. Animals were provided with 12 hours of light/dark cycle [29]. Rats received a standard laboratory diet and ad libitum water. Live animals were used in the study according to the Declaration of Helsinki guidelines on the use of animals in research. Advance ethical approval (ref. GCUF/ERC/254) was obtained from the Ethics Review Committee (ERC) of the Faisalabad University of Government, Pakistan, and the protocols were further authenticated by the Advance Studies and Research Board (ASRB) of the Faisalabad University of Government.

2.5.2. Disease Induction. There are different methods to induce PCOS in the animal model including the estradiol valerate model, testosterone propionate model, DHT (dihydrotestosterone) model, DHAES (dehydroepiandrosterone sulfate) model, and Letrozole induced PCOS model [30]. Among them, letrozole (a nonsteroid agent) was used for this study. It induces morphological and hormonal changes in rats, but metabolic changes are less prominent. Here, in this experiment, the 1 mg/kg (p.o.) dose of letrozole 1 mg/kg (p.o) was used to induce PCOS [31].

2.5.3. Animal Grouping. The animals were randomly divided into 6 groups (each containing 5 rats) and all were marked on the tails. Grouping and dosing details are given in Table 1. The rats were weighed every week. At the end of the 14th week, the rats were weighed and euthanized, and the ovaries were removed to perform histopathology. For analysis of hormonal and liver functioning, blood samples

were collected by cardiac puncture and centrifuged. The serum was separated and used for hormonal assessment and biochemical testing. The liver was preserved for oxidative stress biomarkers [32].

A vaginal smear was also analyzed. Hormonal assays were performed to determine luteinizing hormone, FSH, AMH, progesterone, estrogen, and testosterone levels. A lipid profile test was performed to assess the type (based on quality) and concentration of cholesterol in the blood. Histopathology was performed to analyze the effect of drugs on ovarian histology [33].

2.5.4. Hormonal Analysis. The hormonal assessment was done by using ELISA (enzyme-linked immunosorbent assay) kits, according to protocols provided by the manufacturer. FSH (Lot 02001), LH (Lot 02002), and Testosterone (TES 6511) ELISA kits were purchased from Bio-active Diagnostic Systems JTC Germany. Insulin (Lot INS6770) kits were purchased from Calbiotech, Inc. 1935 Cordel ct, EI Cajon, CA 92020.

2.5.5. Evaluation of an Inflammatory Biomarker. Assessment of inflammatory biomarkers was completed by using ELISA kits purchased from Elabscience, Texas USA. TNF-α (Lot E-ELR0019) and IL-6 (Lot E-ELR0015) ELISA kits were used.

2.5.6. Ovarian Histopathology. The slides are prepared for histopathological analysis. Slides were prepared by fixation of the ovaries in 10% formalin solution and then paraffin blocks were made after weighing, trimming, and embedding the ovaries. The stain used for the dyeing of the slides was hematoxylin and eosin staining (H & E). After staining, the coverslips are placed at the top of the glass slides.

2.5.7. Statistical Analysis. Results were calculated as mean ± SEM. Graph Pad Prism (version 8.4.3) was used to perform the statistical analysis. One-way analysis of variance (ANOVA) was used to compare the results, and multiple comparisons were made with Tukey's post hoc multiple comparison test. Values are considered significant if $P < 0.05$.

3. Results

3.1. Characterization of an Aqueous Ethanolic Extract of Inonotus obliquus (AEIO)

3.1.1. Phytochemical Analysis of AEIO through High-Performance Liquid Chromatography (HPLC). HPLC analysis revealed that the presence of various phytoconstituents in the AEIO were phenolic and flavonoids, including gallic acid, chlorogenic acid, rutin, *p*-coumaric, benzoic acid, quercetin, salicylic acid, and kaempferol. Phytoconstituents with their RT values are shown in Table 2. The HPLC chromatogram is shown in Figure 1. The chemical structures of the phytoconstituents are shown in Figure 2.

3.1.2. Total Flavonoid Content (TFC). The total flavonoid content (TFC) of the *Inonotus obliquus* extract was 18.8%. The TFC is given as the quercetin equivalent (QE).

3.1.3. Total Phenolic Content (TFC). The total phenolic content (TPC) of the *Inonotus obliquus* extract was 30.0%. TPC is given as Gallic Acid Equivalent (GAE).

3.1.4. DPPH Radical Scavenging Assay. The DPPH radical scavenging activity of the hydroalcoholic extract *Inonotus obliquus* was determined. The results of the hydroalcoholic extract were compared with those of the ascorbic acid (standard). The results are shown in Table 3. The AEIO IC_{50} value was 14.21 μg/mL while the ascorbic acid IC_{50} value was 12.98 μg/mL. The results are shown in Figure 3.

3.2. Body Weight Deviations during the Treatment. Throughout the investigation, Letrozole-induced PCOS rats gained significant weight (Table 4). The normal control group showed insignificant weight changes throughout the study. However, AEIO and metformin treatment significantly reduced weight compared to the control group. Results are shown in Table 5.

3.3. Vaginal Smear Cytology. Rats are polyestrous because their estrous cycles go on throughout the year. The proestrus phase is known as the follicular phase and the estrous phase is known as the ovulatory phase. The estrous phase is marked with flat, irregular, and nonnucleated epithelial cells. The metestrus phase usually contains leukocytes and nonnucleated and nucleated cornified epithelial cells. The diestrus phase is marked by many leukocytes and nucleated epithelial cells (Figure 4). Due to the induction of PCOS, the estrus cycle is disturbed and irregulated. It results primarily in a prolonged metestrus phase with abundant leukocytes and nonnucleated cornified epithelial cells (Figure 5).

3.4. Effect of the Aqueous Ethanolic Extract of Inonotus obliquus on the Histopathology of the Ovaries. The ovarian architecture of the normal control group showed normal ovarian histology with corpus luteum, and primary and developing follicles (Figure 6(a)). The slides from the disease group showed an abnormal granulosa cell compartment with cystic follicles and an arrest of growth and lacked corpus luteum (Figure 6(b)). The metformin-treated group showed a reduction in the number of cystic follicles and an improvement in ovarian pathology with the presence of growing follicles, oocytes, and developing follicles (Figure 6(c)). The AEIO 100 mg/kg treated group did not show cystic follicles with few corpus luteum (Figure 6(d)). At the same time, a higher dose of AEIO 300 mg/kg plant extracts showed a reduction in the number of cystic follicles and the presence of corpus luteum along with atretic follicles (Figure 6(e)). The highest dose of AEIO 500 mg/kg almost restored normal ovarian histology with multiple corpus luteum.

Table 1: Dosing schedule during the disease induction and treatment phase.

Sr. #	Groups	Duration (7 weeks)
Dosing schedule during the disease induction phase		
1	Normal control (N.C.)	CMC 0.5% (P.O.) dissolved in CMC was administered
2	Disease control (diseased)	Letrozole (1 mg/kg) dissolved in 0.5% CMC was administered
Dosing schedule during the treatment phase, duration (8^{th}–14^{th} *week)*		
1	Normal control N.C.	CMC 0.5% (po) dissolved in 0.5% CMC was administered
2	Disease control (PCOS)	Letrozole (1 mg/kg) dissolved in 0.5% CMC was administered
3	Standard/metformin 20 mg/kg	Metformin (20 mg/kg p.o.) will be administered to letrozole-induced PCOS rats
4	AEIO 100 mg/kg	Orally administer (100 mg/kg p.o) AEIO to letrozole-induced PCOS rats
5	AEIO 300 mg/kg	Orally administer (300 mg/kg p.o) AEIO to letrozole-induced PCOS rats
6	AEIO 500 mg/kg	(500 mg/kg p.o) AEIO administered to letrozole-induced PCOS rats

CMC: carboxymethylcellulose, AEIO: aqueous ethanolic extract of *Inonotus Obliquus*.

Table 2: HPLC analysis of the aqueous ethanolic extract of *Inonotus obliquus* (AEIO).

No.	Name	R.T.	K. factor	ppm
1	Chlorogenic acid	2.33	0.00013	0.003029
2	Galic acid	3.998	0.000088	0.00035182
3	HB acid	6.976	0.00016	0.00111616
4	Caffeic acid	7.802	0.000059	0.00046032
5	Vanillic acid	7.889	0.000777	0.00612975
6	Kaempferol	12.636	0.0000408	0.00051555
7	Sinapic acid	12.767	0.0000567	0.00072389
8	Ferulic acid	12.873	0.0000767	0.00098736
9	Salicylic acid	15.248	0.000377	0.0057485
10	Coumaric acid	18.341	0.0012	0.0220092
11	Quercetin	23.035	0.000696	0.01603236
12	Benzoic acid	27.031	0.0000345	0.00093257
13	Rutin	28.831	0.000113	0.0032579
14	*p*-coumaric acid	2.728	0.0003	0.0008184

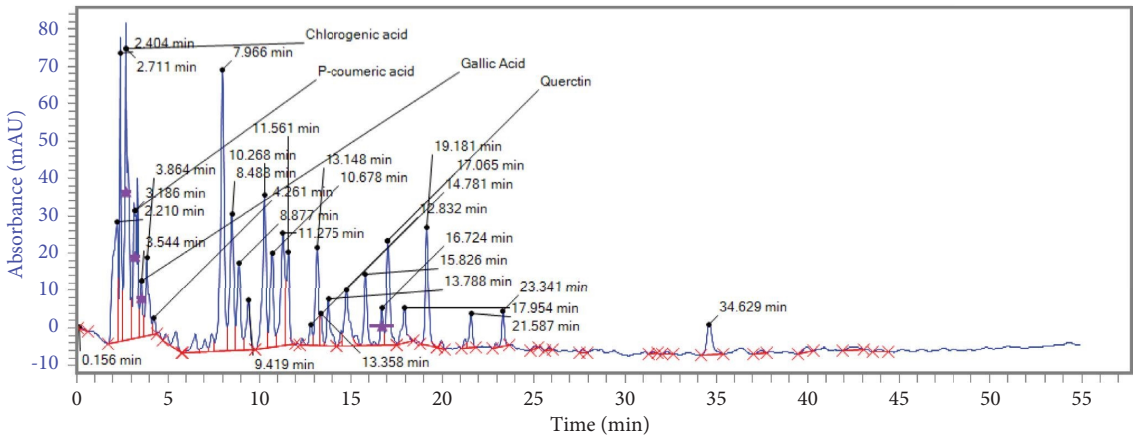


Figure 1: HPLC chromatogram of the ethanolic aqueous extract of *Inonotus obliquus* (AEIO).

3.5. Effect of the Aqueous Ethanolic Extract of Inonotus obliquus on the Hormonal Profile. Induction of PCOS is often associated with hyperinsulinemia that might occur due to insulin resistance (IR). Similarly, in our experiment, PCOS induction resulted in significantly ($p < 0.001$) higher insulin levels. Encouragingly, metformin and AEIO treatment decreased elevated serum insulin levels in a dose-dependent manner. The most significant ($p < 0.001$) effects were observed at the highest dose of AEIO 500 mg/kg. In most PCOS cases, IR causes an increase in LH that could play a role in hormonal imbalance due to disturbance of the gonadotrophin axis. We also observed similar findings; PCOS induction results in significantly higher levels of LH and lower levels of FSH mean elevated LH/FSH ratio. While AEIO significantly ($p < 0.001$) reduced LH and elevated FSH levels, thus restoring the normal LH/FSH ratio. Another

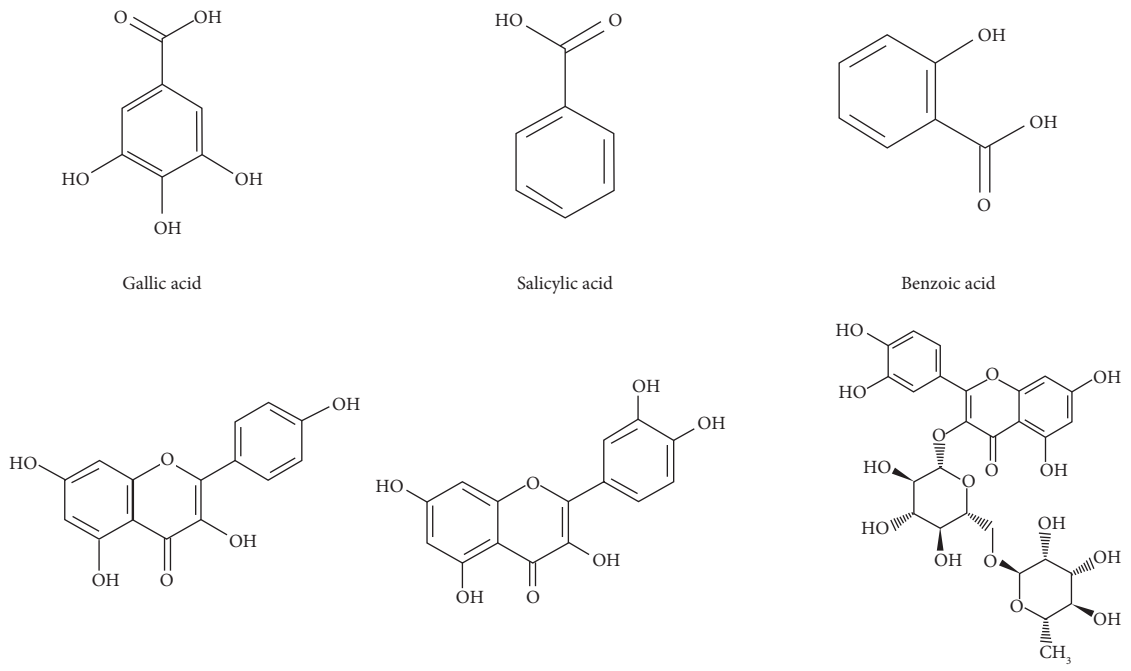


Figure 2: Structural formulas of the phytochemical constituents found in the aqueous ethanolic extract of *Inonotus obliquus* (AEIO).

Table 3: DPPH scavenging activity of the aqueous ethanolic extract of *Inonotus obliquus*.

Sample concentration	Absorbance	Percentage of inhibition (%)
0.48828125	0.48	80.72
0.9765625	0.159	93.61
1.953125	0.153	93.85
3.90625	0.149	94.01
7.8125	0.125	94.97
15.625	0.119	95.22
31.25	0.116	95.34
62.5	0.114	95.42
125	0.112	95.50
250	0.108	95.66
500	0.084	96.62
1000	0.072	97.10

culminating factor in the pathogenesis of PCOS is the excess of androgen in the form of testosterone. When serum testosterone levels were monitored, a significant increase ($p < 0.001$) surge was observed in the disease-induced group compared to the normal control. Encouragingly, treatment with AEIO at all concentrations (100–500 mg/kg) significantly reduced this surge. A nonsignificant effect was observed between the various concentrations of AEIO. In addition, progesterone and prolactin levels were also monitored. PCOS induction perturbed progesterone levels. However, treatment with AEIO and metformin restored normal concentrations. All results are displayed in Figure 7(a)–7(f).

3.6. Effect of Aqueous Ethanolic Extract Inonotus obliquus (AEIO) on the Lipid Profile. PCOS is a metabolic disorder that is often associated with dyslipidemia, which is an abnormality in lipid metabolism. We also expanded our experiment and assessed the lipid profile. The study findings revealed severe hypercholesterolemia in the PCOS-induced group, which is depicted with significantly ($p < 0.05$) higher levels of total cholesterol (TC) along with LDL (low-density lipoproteins) compared to the normal control group. However, AEIO treatment restored not only a normal level of TC but also LDL. The most significant ($p < 0.001$) effects were observed at the 600 mg/kg concentration of AEIO. Similarly, induction of PCOS also caused

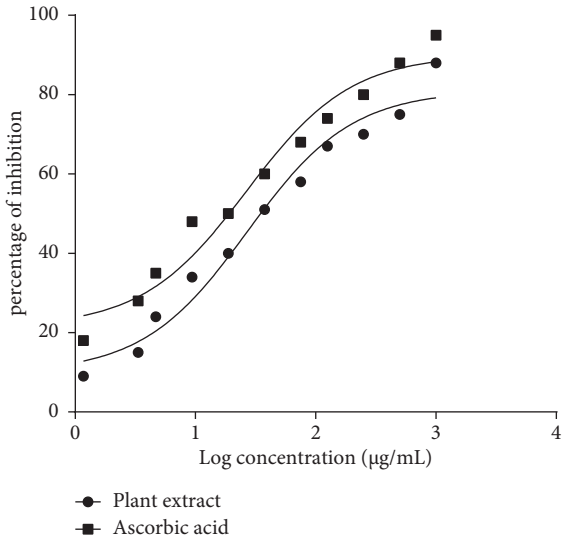


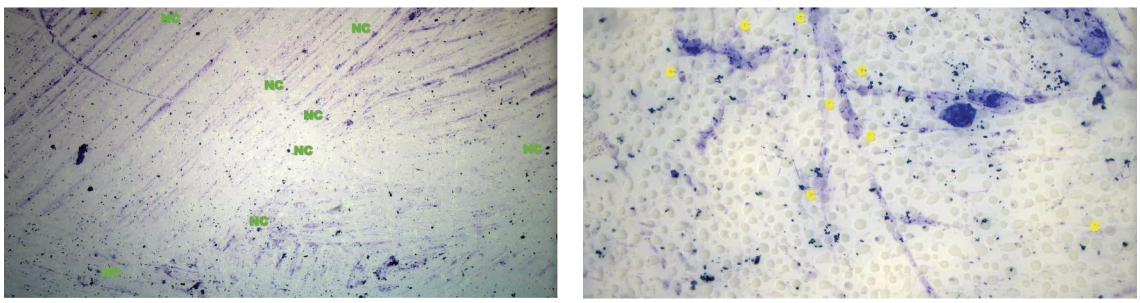
Figure 3: The antioxidant potential of the ethanolic aqueous extract of *Inonotus obliquus* assessed by the DPPH assay.

Table 4: Elevation of weight during the induction phase of the disease.

Groups	Weight elevation during the disease induction phase
Normal control ($n = 5$)	10.07% increase in body weight
Disease control ($n = 25$)	35.02% increase in body weight

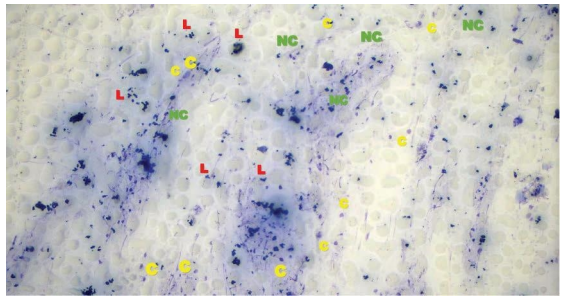
Table 5: Variation of weight during the treatment phase.

Groups	Weight variation during the treatment phase
Normal control ($n = 5$)	7.50% increase in body weight
Disease control ($n = 5$)	22% increase in body weight
Metformin 20 mg/kg ($n = 5$)	22% reduction in body weight
AEIO 100 mg/kg ($n = 5$)	12.9% reduction in body weight
AEIO 300 mg/kg ($n = 5$)	20% reduction in body weight
AEIO 500 mg/kg ($n = 5$)	23.4% reduction in body weight



(a) (b)

Figure 4: Continued.



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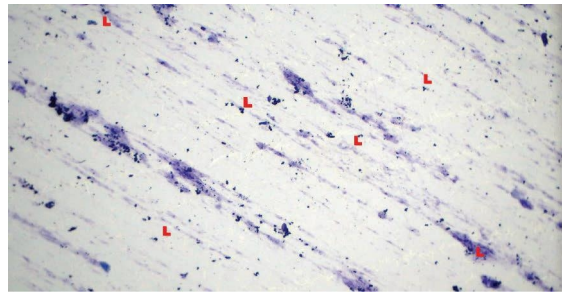


(d)

Figure 4: Vaginal smear cytology of normal rats. (a) The proestrus phase is usually diagnosed with nucleated cornified epithelial cells. (b) Estrus phase with nonnucleated cornified epithelial cells. (c) Metestrus phase containing leukocytes and nonnucleated and nucleated cornified epithelial cells. (d) Diestrus phase: rats have most of the time only leukocytes. NC: nucleated cornified epithelial cells, C: nonnucleated cornified epithelial cells, L: leukocyte.

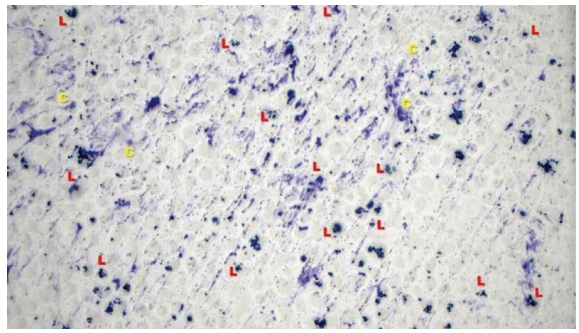
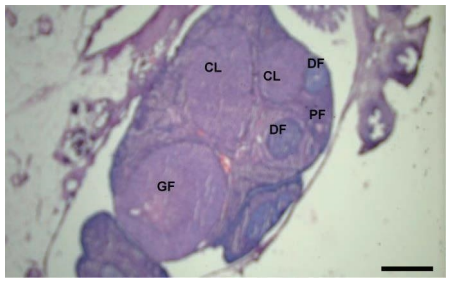
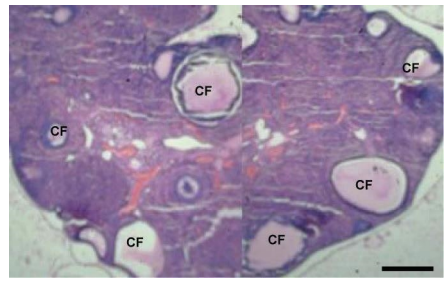


Figure 5: Vaginal smear cytology of diseased (PCOS) rats with a lot of leukocytes and nonnucleated cornified epithelial cells. L: leukocytes, C: nonnucleated cornified epithelial cells.



(a)



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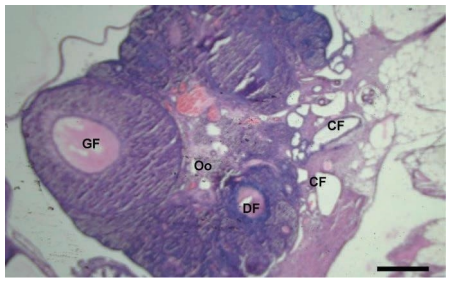

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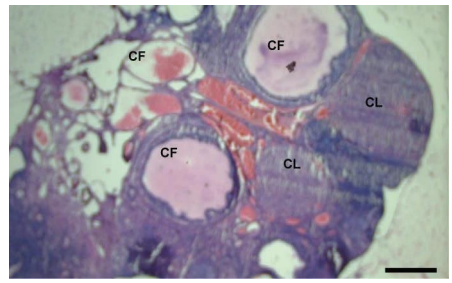
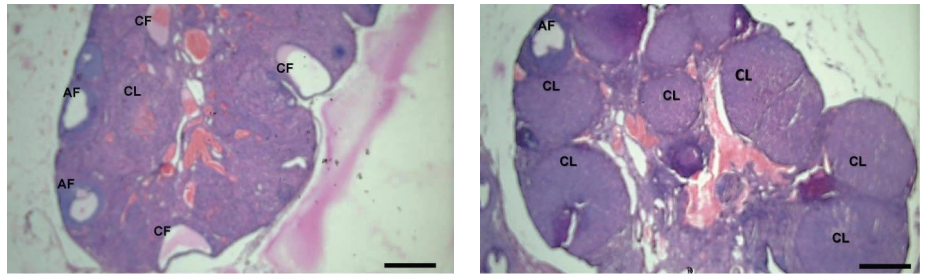


(d)

Figure 6: Continued.



(e) (f)

Figure 6: Cross section of rats' ovaries stained with hematoxylin and eosin. (a) Normal control group section of ovarian morphology showed a normal pattern with corpus luteum and primary and developing follicles. (b) PCOS rats showing cystic follicles. (c) Metformin-treated group showing growing follicles, oocytes, and developing follicles. (d) Group treated with AEIO 100 mg/kg showing corpus luteum and cystic follicles. (e) AEIO 300 mg/kg treated group ovarian cross section showing cystic follicles and atretic follicles. (f) The AEIO 500 mg/kg treated group exhibited normal morphology with corpus luteum. AF: atretic follicles, CF: cystic follicles, CL: corpus luteum, GC: granulosa cells, DF: developing follicles, GF: growing follicles, Oo: oocyte. The magnification used was 4×. The scale bar was 100 μM.

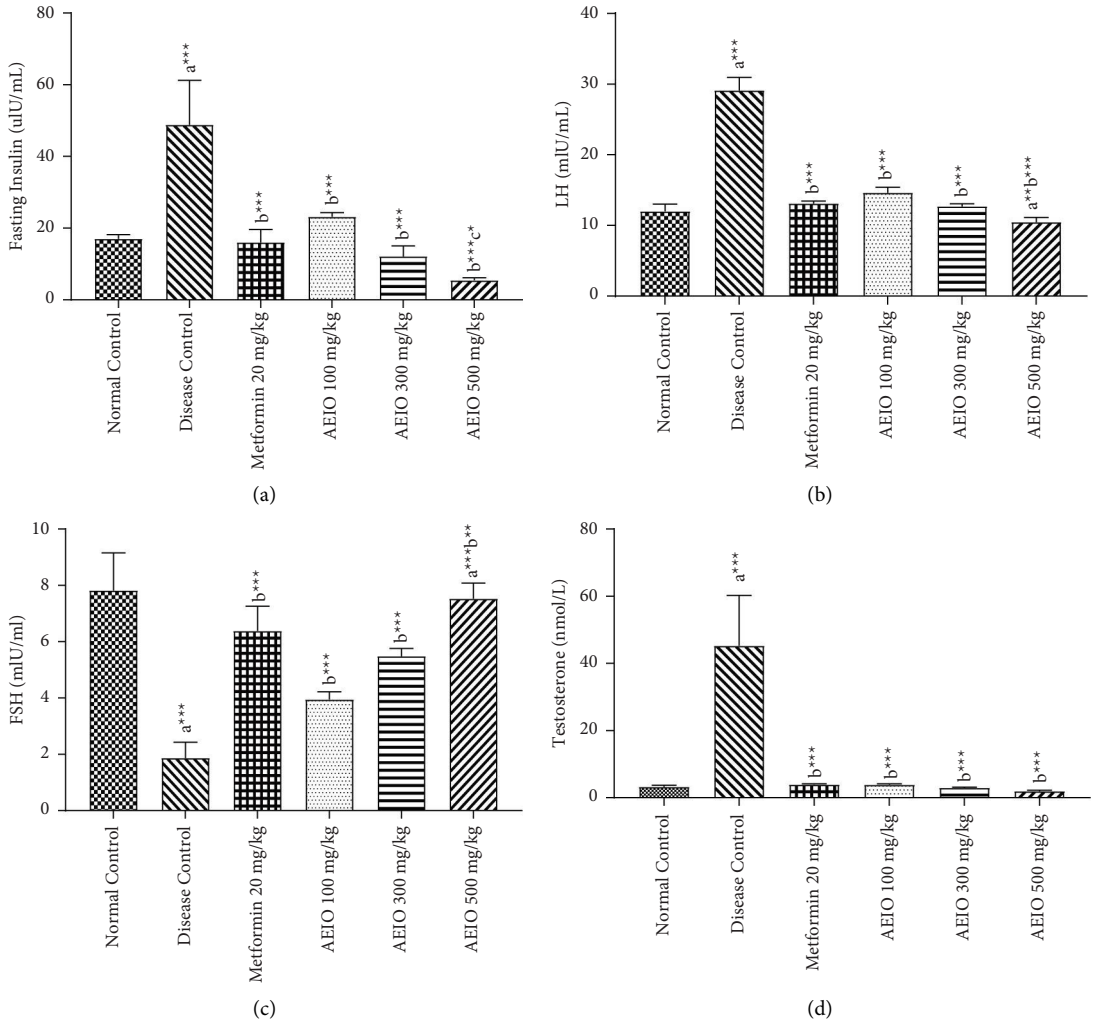


Figure 7: Continued.

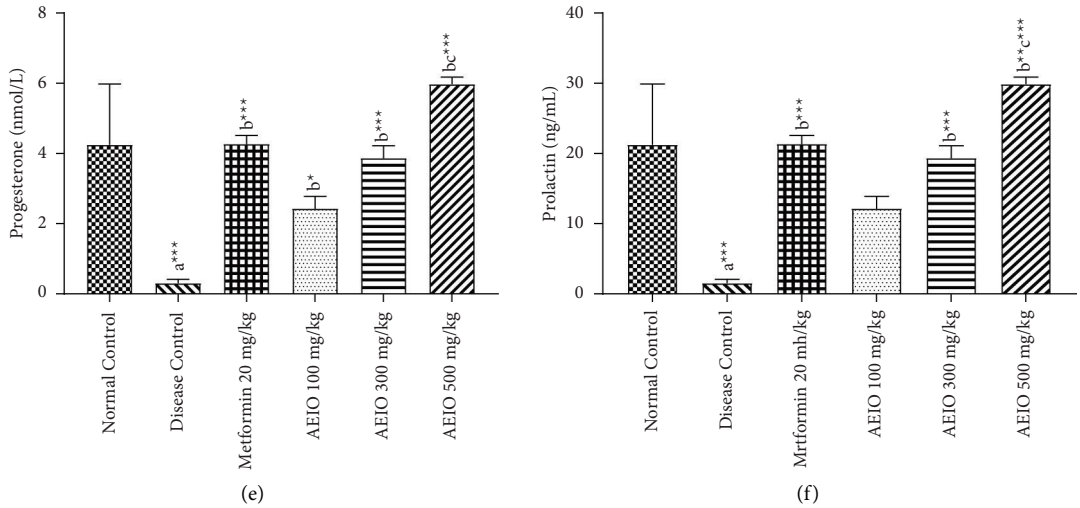


Figure 7: Effect of the aqueous ethanolic extract of *Inonotus obliquus* (AEIO) on (a) insulin, (b) LH, (c) FSH, (d) testosterone, (e) progesterone, (f) prolactin level in the letrozole-induced PCOS rat model. Values are expressed as mean ± SEM (n = 5). Groups were statistically compared using one-way analysis of variance (ANOVA), and multiple comparisons were performed using Tukey's multiple comparison test. A represents a significant difference from the normal control group. B represents a significant difference from the disease control group. C represents a significant difference from the AEIO 100 mg/kg group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

hypertriglyceridemia depicted with abnormal TG levels in the PCOS group, but AEIO treatment normalized it. AEIO effects were concentration-dependent. Results are shown in Figure 8.

3.7. Effect of Aqueous Ethanolic Extract Inonotus obliquus (AEIO) on Oxidative Stress and Its Role in Restoring Hormonal Balance and Ovarian Histopathology. In the upper sections of our study, we have found that PCOS induction is closely related to IR, hyperinsulinemia, hyperandrogenism, and abnormal LH/FSH with multiple cystic follicles in the ovaries in animals in the disease-induced group. Thus, to affirm the mechanism behind these pathological events in ovaries, we determined oxidative stress (OS) and reactive oxygen species (ROS) production. Because the data already published indicated the role of oxidative stress in the pathogenesis of PCOS, ROS and SOD (superoxide dismutase) played an important role in ovulation [34]. The findings of our study showed that PCOS induction significantly ($p < 0.001$) lessened the SOD level. Treatment with AEIO at various concentrations (100, 300, and 500 mg/kg) produced a positive impact on ovarian pathology with an elevated level of SOD. These effects were dose-dependent, and the most significant effect ($p < 0.001$) was observed at AEIO 500 mg/kg (Figure 9(a)). CAT (catalase) is another important enzyme that hydrolyzes hydrogen peroxide, which means that it plays a pivotal role in reducing the ROS burden. PCOS induction significantly ($p < 0.001$) reduced their levels, but AEIO restored their normal levels (Figure 9(b)). GSH (glutathione) is another antioxidant enzyme that is also a protective barrier against OS. Previous studies indicated its significantly lower levels in PCOS [35, 36]. Similarly in our study, PCOS induction significantly reduced ($p < 0.001$) the concentration of GSH. AEIO increased GSH concentration in a dose-dependent fashion (Figure 9(c)). MDA is an extensively studied lipid peroxidation marker and its higher levels are closely associated with the pathogenesis of PCOS [37]. Higher levels of MDA were observed in the PCOS group; however, AEIO significantly restored their normal concentrations, as shown in Figure 9(d).

3.8. Effect of Aqueous Ethanolic Extract Inonotus obliquus (AEIO) on Inflammatory Cytokines Expression. As already mentioned, PCOS pathogenesis has a strong link with oxidative stress (OS). OS initiates low-grade inflammation, which is depicted by the upregulation of NF-κB expression, along with the expression of various inflammatory cytokines such as TNF-α, and IL-6 [38, 39]. Our study findings also revealed similar findings with significant ($p < 0.001$) overexpression of TNF-α and IL-6 in the PCOS group compared to the normal control. AEIO treatment significantly ($p < 0.01$) downregulated both overexpressed cytokine levels (Figure 10).

4. Discussion

PCOS is an endocrine and metabolic abnormality, and its prevalence is increasing day by day due to numerous factors. It is a disorder of young age and is most of the time associated with infertility [40]. Characteristic features of PCOS are IR, hyperandrogenism, and hypercholesterolemia [41]. Hormones are important regulators of the normal physiological functions of the body. Follicular maturation is an integral part of the normal menstrual cycle which culminates in ovulation. The normal physiological function of the ovary along with the optimal level of GnRH, LH, and FSH levels are essential. In PCOS, the follicle does not mature and ovulate because drastic pathophysiological changes occur in the ovaries. There are abnormalities in the completion of

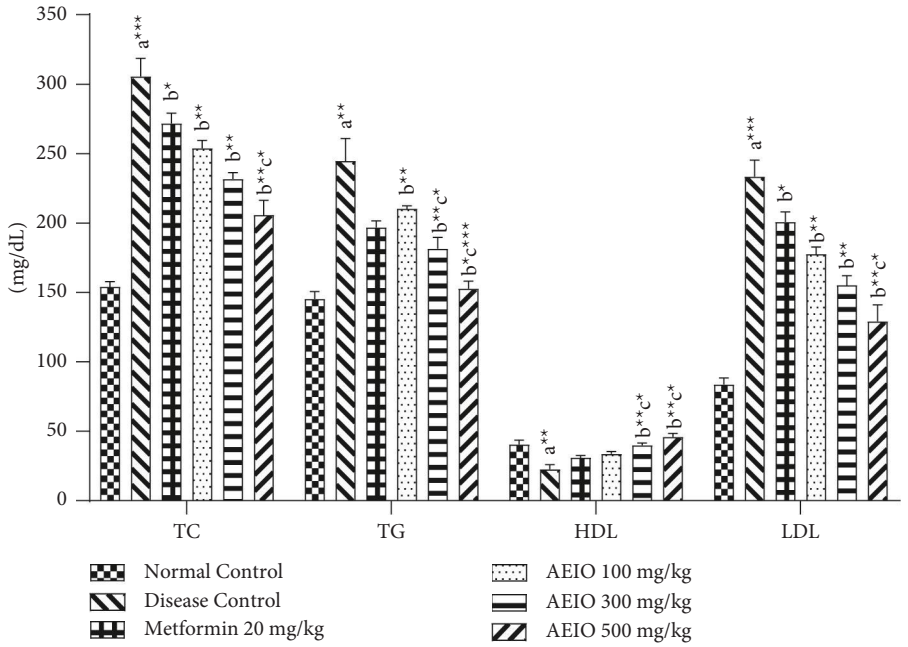
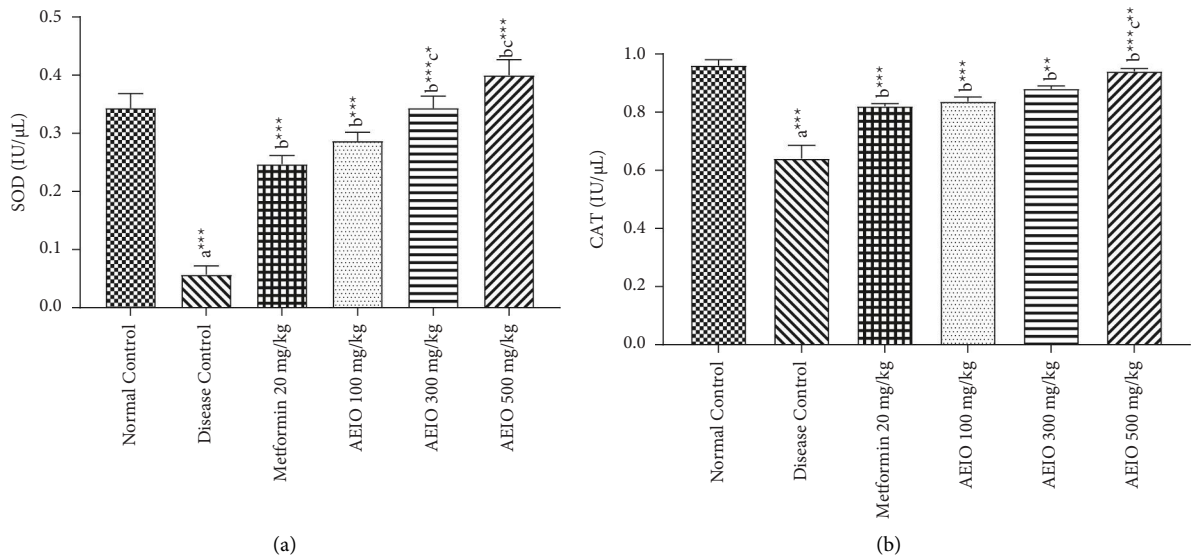


Figure 8: Effect of the aqueous ethanolic extract of *Inonotus obliquus* (AEIO) on the lipid profile in the letrozole-induced PCOS rat model. Values are expressed as mean ± SEM ($n = 5$). Groups were statistically compared using one-way analysis of variance (ANOVA), and multiple comparisons were performed using Tukey's multiple comparison test. A represents a significant difference from the normal control group. B represents a significant difference from the disease control group. C represents a significant difference from the AEIO 100 mg/kg group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. TC: total cholesterol, TG: triglyceride, HDL: high-density lipoprotein. LDL: low-density lipoprotein.



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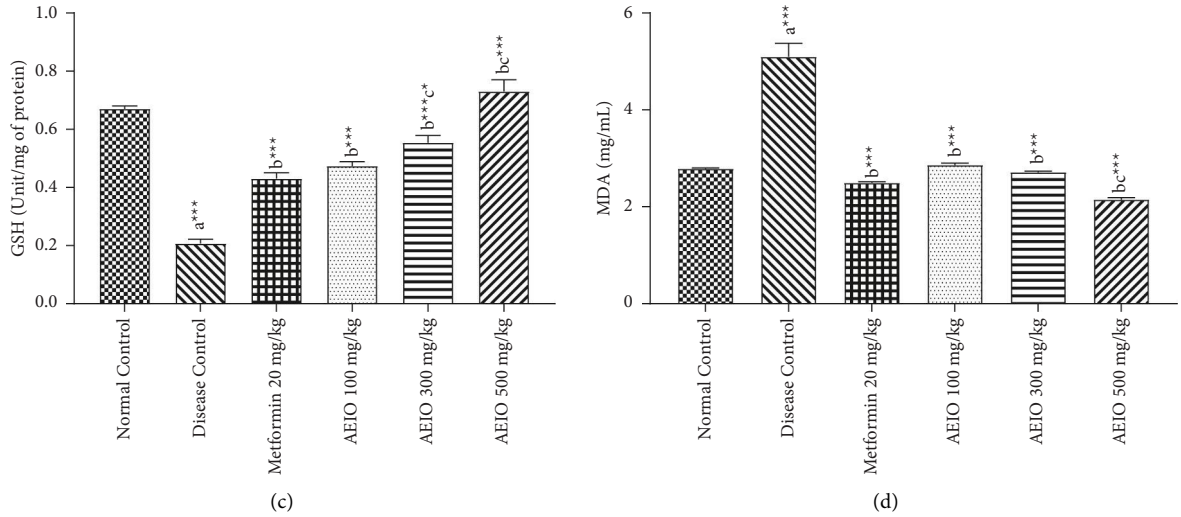

Figure 9: Effect of the aqueous ethanolic extract of *Inonotus obliquus* (AEIO) on oxidative stress in the letrozole-induced PCOS rat model. Values are expressed as mean ± SEM ($n = 5$). Groups were statistically compared using one-way analysis of variance (ANOVA) and multiple comparisons were performed using Tukey's multiple comparison test. A represents a significant difference from the normal control group. B represents a significant difference from the disease control group. C represents a significant difference from the AEIO 100 mg/kg group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

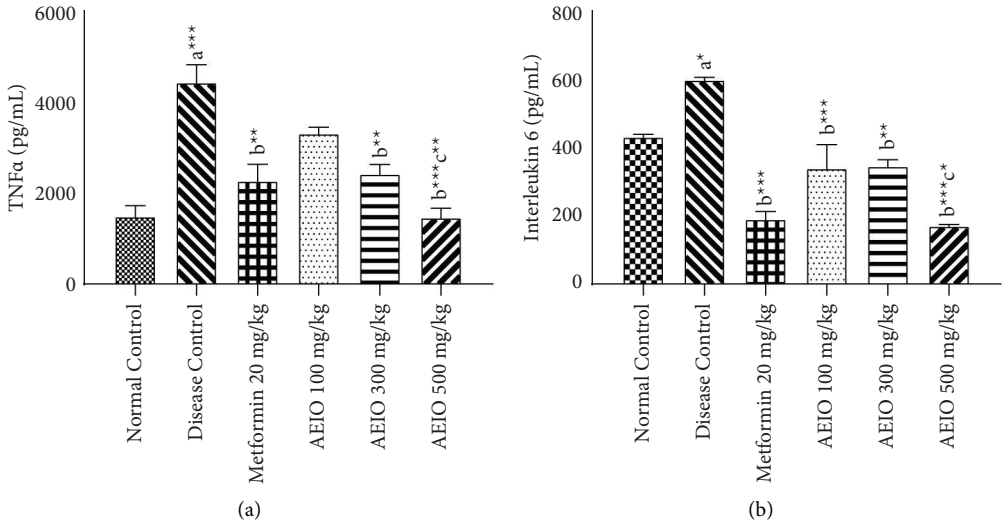

Figure 10: Effect of the aqueous ethanolic extract of *Inonotus obliquus* (AEIO) on inflammatory cytokines; TNF-α and IL-6 in the letrozole-induced PCOS rat model. Values are expressed as mean ± SEM ($n = 5$). Groups were statistically compared using one-way analysis of variance (ANOVA), and multiple comparisons were performed using Tukey's multiple comparison test. A represents a significant difference from the normal control group. B represents a significant difference from the disease control group. C represents a significant difference from the AEIO 100 mg/kg group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

both the follicular and luteal phases. The main cause behind this vicious cycle is not known, but defective checkpoints in PCOS are disturbances in hormonal regulation and genetic defects [7, 8].

These pathophysiological changes lead to infertility, irregular menstruation, diabetes, and heart disease. Allopathic treatment regimens including ovulation inducers such as citrate, letrozole, and metformin are used mainly. In addition, oral contraceptive protocols (OCP) are used for menstrual abnormalities. Unfortunately, none of these treatments are devoid of adverse effects. The possibility of developing causative treatment is not as certain. People are more inclined towards natural herbal remedies to treat PCOS due to their lower tendency to adverse effects. The chaga mushroom is quite popular for its uses. It is a powerful pack of highly potent phytochemicals. It is a densely rich source of flavonoids and phenolic compounds, including botulin, botulinic acid, ergosterol, gallic acid, kaempferol,

benzoic acid, rutin, chlorogenic acid, p-coumaric acid, salicylic acid, and quercetin. All of them possess distinct properties [19, 21, 23].

Letrozole (1 mg/kg), an aromatase inhibitor, was used to induce PCOS [42, 43]. It induces pathophysiological PCOS conditions such as human PCOS in terms of cystic follicles and dysregulation of hormones. Letrozole is a competitive aromatase inhibitor that binds to the heme region of this enzyme. Aromatase is responsible for the synthesis of estrogens, progesterone, and androgens. It also converts androgens to estrogens. By inhibiting this enzyme, reduced estrogen levels send negative feedback to the hypothalamus, thereby altering the release of GnRH. GnRH regulates the release of gonadotropin hormones. Therefore, letrozole dysregulates the functioning and concentration of androgens, insulin, LH, and FSH [11, 44]. These hormones are of primary importance in the normal menstrual cycle and ovulatory mechanisms. In addition, they inhibit the normal discharge of the corpus luteum, causing it to form cysts in the ovaries. This study aimed to explore the pharmacological potential of the aqueous ethanolic extract of *Inonotus obliquus* (AEIO) against letrozole-induced PCOS and to investigate its molecular mechanism. HPLC analysis of AEIO revealed the presence of various phytochemicals such as gallic acid, chlorogenic acid, rutin, *p*-coumaric, benzoic acid, quercetin, salicylic acid, and kaempferol (Figures 1 and 2).

PCOS induction is confirmed by various methods; two important methods are weight gain and vaginal smear cytology. We observed a significant increase in weight gain in PCOS rats which was 35.02% as compared to the normal control group in which the increase was 10.05% (Table 4). The elevation in body weight among diseased rats can be explained by the positive correlation between androgenic excess along with reduced sensitivity to insulin. These findings were consistent with the published data [45]. The second parameter used for disease induction was vaginal smear cytology. In PCOS, the diestrus phase is prolonged and the estrous phase is delayed [45]. We have observed irregular cyclicity of the estrus cycle and an abundance of leucocytes and nucleated epithelial cells in disease-induced rats which indicated a prolonged metestrus phase and a delayed estrus phase (Figures 4 and 5). These results are consistent with already published data [46]. As already discussed, weight gain is an important indicator of PCOS induction. We observed the effect of AEIO and metformin on the weight of PCOS rats and observed that AEIO treatment, especially the concentration of 500 mg/kg, significantly reduced the weight (24% reduction) of rats compared to the control group (7.5% increase). Similar findings were also observed with metformin treatment (Table 5).

Ovarian morphology and ultrasound are the main diagnostic features of PCOS based on Rotterdam criteria. The presence of cystic follicles and hyperandrogenism are characteristic features of PCOS [40]. We performed ovarian histopathology which revealed the presence of cystic follicles in PCOS rats, which is a characteristic of PCOS induction. AEIO treatment improved ovarian pathology with the presence of growing follicles, oocytes, developing follicles, and corpus luteum (Figure 6(a)–6(e)). Therefore, the findings of the study indicated restoration of ovarian morphology and alleviation of the pathological abnormalities associated with PCOS. Furthermore, a hormonal evaluation was carried out and hyperinsulinemia, hyperandrogenism, increased LH, and lower levels of FSH were found in female rats with PCOS. However, AEIO significantly restored normal levels of all the above-mentioned hormones in a dose-dependent manner (Figure 7(a)–7(f)).

Hyperandrogenism (elevated level of androgens) is a prominent feature of PCOS. The most predominant androgen is testosterone. Hyperandrogenism (HA) and PCOS are interlinked. HA is responsible for many signs and symptoms of PCOS, such as hirsutism, acne, alopecia, IR, and menstrual abnormalities. These androgens are not only produced by the ovaries but also by the adrenal cortex in females, and include androgen metabolites and 11 oxygenated androgens. Among them is 11-ketotestosterone (11keto T), which might be equipotent to testosterone. This might happen due to aldo-keto reductase family 1 member C3 (AKR1C3) [47, 48]. 11-keto T is not metabolized by aromatase and cannot be converted into estrogen. Importantly, 11-ketoT is a nonaromatizable androgen, cannot be converted to estrogen, and has higher bioavailability because it has a low affinity for SHBG (sex hormone-binding globulin). LH surge is also associated with elevated androgen production. LH in synergy with hyperinsulinemia stimulates theca cell growth to reduce SHBG production. Androgen excess promotes IR and inflammation, leading to ROS production and oxidative stress (OS). OS elevated NF-κB expression which is responsible for inflammatory episodes by provoking upregulation of TNF-α and IL-6. This inflammation leads to upregulation of CYP-450 17 α hydroxylase, thus the production of excessive androgen in the ovaries. The state of OS (oxidative stress) worsens when the production of SHBG decreases [49]. This affects insulin signaling pathways within cells, leading to IR. Furthermore, IR and hyperinsulinemia contribute to OS and vice versa. IR is also a major cause of hyperglycemia (high blood sugar levels) [50].

Oxidative stress could be assessed by measuring various enzymatic markers that directly played an active role. SOD (superoxide dismutase) is an important enzyme that catalyzes the conversion of superoxide radicals into less harmful molecular oxygen and hydrogen peroxide. The superoxide radical is an important element of OS [51]. CAT (catalase-peroxidase) is another important antioxidant enzyme that neutralizes hydrogen peroxide, thus regulating its concentration to prevent OS and maintain normal signaling pathways within the cells [52]. GSH (glutathione) is a strong antioxidant that protects cells from OS, ROS, lipid peroxides, and free radicals [53]. Malondialdehyde (MDA) is produced because of lipid peroxidation, and it is used as a marker of oxidative stress [54]. Considering the above discussion, we determined the level of SOD, CAT, GSH, and MDA. Our findings revealed that PCOS induction significantly decreased the level of SOD, CAT, and GSH while upregulating the level of MDA (Figure 9). Thus, we found a clear picture of severe OS and ROS production. Oxidative

stress (OS) initiated a vicious cycle, which further exaggerated IR, hyperinsulinemia, hyperandrogenism, cystic follicles, and ovulation arrest. This process culminates in an inflammatory cascade with the expression of NF-κB and its associated cytokines TNF-α and IL-6 (Figure 10). AEIO significantly restored the level of SOD, CAT, and GSH, and decreased MDA levels in a dose-dependent manner, thus putting a check on OS and ROS production. Furthermore, AEIO decreased the expression of TNF-α and IL-6, thus hindering the inflammatory cascade which was the crucial part of this oxidative stress and the pathogenesis of the disease.

5. Conclusions

AEIO has been shown to alleviate PCOS symptomatology and improve the abnormalities associated with the disease. It contains phytochemicals such as gallic acid, chlorogenic acid, rutin, p-coumaric, benzoic acid, quercetin, salicylic acid, and kaempferol, which restored normal levels of natural antioxidants SOD, CAT, and GSH. In addition, it inhibited oxidative stress (OS) and reduced the production of reactive oxygen species (ROS). Furthermore, AEIO has been found to downregulate the expression of inflammatory cytokines TNF-α and IL-6 associated with NF-κB which were responsible for low-grade inflammation associated with the disease. These findings are significant for the less deprived populations who have scant access to modern-day facilities and rely on herbal remedies. They provide a scientific ground wherein traditional treatment modalities can be linked with modern-day clinical approaches.

A limitation of the study was that we were unable to find molecular data, especially at the genetic level. However, this study provided a platform on which base molecular studies can be initiated.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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