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# ASSESSMENT OF *OCIMUM SANCTUM* TO NORMALIZE THE ESTROUS CYCLE IN LETRAZOLE INDUCED POLYCYSTIC OVARY SYNDROME IN FEMALE WISTAR RATS

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## ABSTRACT

**Objective:** To assess beneficial effect of ethanol leaf extract of *Oscimum Sanctum* in Letrozole induced Poly cystic ovary syndrome in female Wistar rats. **Methods:** Letrozole (1 mg/kg) was administered per orally (p.o) for a period of 21 days for the induction of PCOS, followed by doses of extract (100 mg/kg and 200 mg/kg, p.o) for 15 days using carboxy methyl cellulose(CMC) as vehicle. **Results:** The administration of Letrozole led to abnormalcy in serum sex steroid profile, glucose and depletion in antioxidant activity. Leaf Extract of *Oscimum sanctum* was able to successfully exert its protective effect by restoring all the parameters to normal and disappearance of cysts in ovaries. **Conclusion:** Leaf Extract of *Oscimum sanctum* showed beneficial effects in Letrozole induced PCOS in female Wistar rats. Its effect was comparable to that of Clomiphene citrate, most widely used treatment for ovulation induction in PCOS condition.

**KEYWORDS:** PCOS, Letrazole, Oscimum sanctum, Clomiphene citrate.

## 1. INTRODUCTION

Polycystic ovary syndrome (PCOS) is an endocrine disorder characterized by anovulation, amenorrhea, hirsutism and infertility. It is also known as stein Levithel syndrome.[1] A total of 1–5% of women suffer from PCOS and the incidence appears to be on the increase due to

changes in lifestyle and stress.[8] Long term consequences lead to cancer, type-II diabetes mellitus, dyslipidemia, hypertension, and cardiovascular disorder. The etiology of PCOS is not clearly understood, but lipid imbalance, oxidative stress, insulin resistance and genetics are some of the contributing factors.[12] The exact pathophysiology of PCOS are uncertain, evidence suggests that an excess of ovarianandrogen production, either genetically or due to extra ovarian factors such as hyperinsulinemia or disturbances of the hypothalamic–pituitary–ovarian axis is the main cause in the pathogenesis of PCOS.[1,4] Although various short term symptomatic therapies are available, the best long term management strategies have not been recognized.

Letrozole, a non-steroidal aromatase inhibitor produces a PCOS model which in numerous ways depicts human PCOS. It blocks conversion of testosterone and androstenedione to estradiol and estrone respectively and simulates PCOS like condition by causing hormonal imbalance, circulating hyperandrogenism and intra ovarian androgen excess leading to appearance of polycystic ovary. Follicular atresia and abnormal follicular development is observed due to induced elevation of androgen levels inside the ovary. Letrozole induction was reported to cause hyperglycaemic condition which may contribute to insulin resistance, hyperlipidemia leading to metabolic syndrome.[5,7]

Herbal plant drug form main source of health care due to more effectiveness, lower cost and well tolerated by the patient having fewer unintended consequences and fewer side effects than traditional medicine and may be safer to use. Herbal plants have been used since centuries to correct disorders caused by the hormonal imbalance related to female reproductive system. Oscimum Sanctum is also known as Tulsi and holy basil has been used for the treatment of a wide range of ailments in many parts of the world. This Plant possess strong anti-inflammatory, analgesic, antipyretic, antidiabetic, hepatoprotective, hypolipidemic, antistress, and immunomodulatory activities etc. there is no evidence for the beneficiary effect of this plant on letrazole induced pcos.

## 2. MATERIALS AND METHODS

### 2.1. Experimental animals

Virgin, cyclic, adult female Wistar Albino rats (160–200 g) were employed for the study. Animals were acquired from Animal house of Teena Labs, Kukatpally, Hyderabad and housed in our institution's animal house and allowed to acclimatise for two weeks. During the study all animals were caged in standard polypropylene cages and maintained in controlled

environment of (22 ± 3) _C temperature, (55 ± 5)% humidity and a 12 h light/ dark cycle. They were fed with standard diet and water provided ad libitum. The study was duly approved by Institutions Animal Ethics Committee for the use of animals and care of the animals was carried out as per the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA).

### 2.2. Drugs and reagents

Letrozole was obtained from Natco Pharma Limited, Hyderabad. Clomiphene Citrate (Fertyl-Super) tablets were procured from Ar-Ex Laboratories Private Limited, Goregaon (E), Mumbai. All other chemicals used were of analytical grade. The Glucose, Cholesterol, Triglycerides, HDL and Glycosylate Hemoglobin kits were obtained from ERBA Diagnostic, USA.

### 2.3. Plant material

The leaves of *Oscimum sanctum (OS)* were collected from the Botanical garden of Anurag Group of Institution, Ghatkesar, India. The drug was authenticated by Dr.Ram Mohan, Associate Professor, Pharmacognosy. The test drug was cleaned from impurities and coarse powder was made with electrical grinder, 100 g of which was used for extraction in ethanol as a solvent for approximately 6 hours by using Soxhlet apparatus (Borosil glass works Ltd. Gujrat, India) at 60◦C. The liquid extract was cooled and filtered by filter paper (What-man no. 40) and then evaporated on a water bath (60◦C) until it dried completely. The yield percentage of extract was found to be 25% w/w (Table 1).

### 2.4. PCOS induction

All the experimental animals except control group, were orally administered with Letrozole at a dose of 1 mg/kg dissolved in 0.5% Carboxy Methyl Cellulose (CMC) once daily for 21 days [KAFAI]. Control group received vehicle only (0.5% CMC). Vaginal Smears were collected daily and evaluated microscopically using Giemsa stain to confirm the induction of PCOS.[11,12]

### 2.5. Study design

The study consisted of 30 female Albino Wistar rats equally divided into five groups designated as group 1 (served as control group), group 2 (served as PCOS induced group), group 3 (served as standard group), groups 4 and 5 served as treatment groups. Following Letrozole administration, standard group was administered with Clomiphene Citrate at a dose

of 1 mg/kg in 0.5% CMC per oral and treatment groups 4 and 5 were administered Extract with the dose of 100 mg/kg (Low dose) and 200 mg/kg (High dose) body weight respectively in 0.5% CMC per oral for 15 days i.e., from day 22 to day 36. After 21 days, PCOS control group and after 36 days, animals from other groups were fasted overnight and anaesthetized with diethyl ether. Blood was collected by retino orbital puncture then serum was separated by centrifugation and was used for estimation of hormones, glucose and lipid parameters. The animals were then sacrificed, ovaries and uterus excised, cleaned of fat and weighed. After excision, ovaries were freed from blood and cleaned with ice cold saline and homogenized using10% ice cold potassiumchloride for antioxidant evaluation.[8,10]

### 2.6. Phytochemical analysis

The extract of OS was subjected to preliminary screening of phytochemicals such as alkaloids, flavonoids, phenols, tannins, saponins, sterols, and glycosides.[7]

### 2.7. Biochemical estimations

#### 2.7.1. Hormonal assay

Hormones were assayed by Competitive Chemiluminescent Immunoassay using automated instrument ADVIA Centaur, Siemens Healthcare Diagnostics Inc., USA. The testosterone was estimated using ADVIA Centaur TSTO kit, estrogen using ADVIA Centaur E2-6 kit, and progesterone using ADVIA Centaur PRGE kit.

#### 2.7.2. Measurement of fasting blood glucose (FBG)

FBG was measured by Trinder's method using a commercial diagnostic kit from ERBA Diagnostics, USA.

#### 2.7.3. Assessment of lipid profile

Lipid profile [total-cholesterol (TC), triglycerides (TG), and HDL-cholesterol (HDL-C)] were estimated by using enzymatic kits procured from ERBA Diagnostics, USA. LDL-cholesterol (LDL-C) was calculated by using Friedewald's equation.

#### 2.7.4. Antioxidant assay

##### 2.7.4.1. Superoxide dismutase

Superoxide dismutase activity was determined by the pyrogallol oxidation method. This is an indirect method that is based on the ability of the enzyme to inhibit the auto-oxidation of pyrogallol. Superoxide dismutase activity was determined by monitoring the rate of oxidation

of pyrogallol by superoxide radicals. The reaction is initiated by adding pyrogallol and the change in optical density was recorded at 420 nm[1].

#### 2.7.4.2. Catalase

Catalase activity was determined in 50 ml of sample mixed with 50 ml of substrate for 60s, then 100 ml of 32.4 Mm ammonium molybdate solution was added and absorbance change was measured at 405 nm. One unit of the enzyme was defined as mmoles of H2O2 degraded/min/mg of protein [ALAM].

### 2.8. Ovarian histomorphology

Excised ovaries were fixed in 10% Neutral Buffered Formalin. They were subjected to tissue processing by dehydration through an ascending ethanol series, clearing in xylene and embedding completely in paraffin wax into blocks. The blocks were then serially sectioned at 5 mm thickness using microtome and were mounted on poly-lysine coated slides, deparaffinised using xylene, rehydrated and stained with hematoxylin and eosin, dehydrated, cleared and mounted on DPX under glass cover slips. The slides were then observed under light microscope connected to a camera to capture images.

### 2.9. Statistical analysis

The data was statistically analyzed using one-way ANOVA followed by Newman–Keuls multiple comparison tests and expressed as mean ± standard error of mean. $P < 0.05$ was considered to be statistically significant. The statistical analysis was carried out with Graph pad prism 5.0 software.

## 3. RESULTS

### 3.1. Physicochemical constituents

Ethanolic leaf extract of OS has alkaloids, saponin, phenols, flavonoids, glycosides and tannins was given in table 1.

### 3.2. Effect of Extract on Serum hormonal profile

The serum levels of Testosterone were remarkably increased in PCOS induced group ($P < 0.001$) while those of Progesterone and Estradiol decreased significantly ($P < 0.001$ and $P < 0.001$, respectively) in comparison to the control group. A significant fall ($P < 0.001$) in testosterone levels was observed in standard, low dose and high dose groups (table 2). Progesterone levels were also increased significantly ($P < 0.001$) in all the treatment groups

i.e., in groups 3, 4 and 5. Standard group and high dose group showed significant increase ($P < 0.001$) and low dose showed significant increase ($P<0.01$) in estradiol levels when compared to PCOS induced group.

### 3.3. Effect of Extract on FBG

Figures 2 interpret the effect of Extract on serum glucose. PCOS induced group showed significant increase in glucose level ($P < 0.001$) in comparison to control group. All the treatment groups exhibited significant decrease in levels of glucose parameters ($P < 0.001$) when compared to PCOS induced group.

### 3.4. Effect of Extract on serum lipid profile

Letrozole treatment caused significant changes in serum lipid as compared to control. TG's, TC and LDL were greatly increased as $P < 0.001$, $P < 0.001$ and $P < 0.001$ respectively while HDL levels were notably decreased ($P < 0.001$) in PCOS induced group. Table 3 portrays effect of Extract on lipid profile. Clomiphene treatment significantly decreased TG ($P < 0.001$), TC ($P < 0.001$) and LDL ($P < 0.001$) levels when compared to PCOS induced group. Low dose of Extract decreased the levels of TG ($P < 0.001$), TC ($P < 0.001$), LDL ($P < 0.01$) and HDL ($P<0.01$) significantly. High dose of Extract significantly reduced TG, TC and LDL levels($P < 0.001$). It also increased HDL levels significantly ($P < 0.001$) in comparison to PCOS induced group.

### 3.5. Effect of Extract on antioxidant activity

Table 3 illustrates effect of Extract on antioxidant activity and lipid peroxidation. PCOS induced group showed conspicuous depletion in antioxidant enzyme activity SOD ($P < 0.001$) and Catalase ($P < 0.001$) (table 4). Standard group show significant increase in SOD ($P<0.05$) Catalase ($P < 0.01$) activity close to those in the control group. Low dose (100 mg/kg) and high dose(200mg/kg)showed its after effect by increasing the activity of SOD ($P < 0.001$) and Catalase ($P < 0.001$) when compared to control group [alam].

### 3.6. Histomorphological changes

Sections of ovaries from control group animals showed healthy follicles with oocyte at different stages of development (Figure 3). Letrozole treated rats exhibited numerous subcapsular cysts, with a very thin or no granulosa layer (Figure 4). Corpora lutea were completely absent indicating anovulation. Few follicles were observed at their early stages of development. In addition, they were accompanied with atretic follicles containing fluid filled

antrum and higher incidence of pyknotic granulosa cells. Clomiphene citrate treatment led to disappearance of cysts and appearance of healthy follicles and corpora lutea. Sections from low dose of low dose (100 mg/kg) group exhibited follicles larger in size and few corpora lutea (Figure 5). Cysts were absent and normal sized healthy follicles at different developmental stages with oocytes were found in section from high dose (200 mg/kg) group (Figure 6). Also with the high dose many corpora lutea and antral follicles with clearly differentiate oocyte, granulosa cell layer, corona radiate, cumulus oophorus and thecal cells were observed.

**Table 1: Phytochemical Analysis of Ethanol leaf Extract of *Oscimum Sanctum*.**

| Constituents | Ethanolic leaf Extract of OS |
|---|---|
| Alkaloids | + |
| Saponin | + |
| Phenols | + |
| Flavonoids | + |
| Glycosides | + |
| Tannins | + |

**Table 2: Effect of various treatments on Serum Sex steroids.**

| Group | Testosterone (ng/dl) | Progesterone (ng/dl) | Estradiol (pg/dl) |
|---|---|---|---|
| Control | 35.2±8.58 | 27.8±1.92 | 26.8±0.83 |
| PCOS Control | 147.4±4.15$^{a***}$ | 13.0±1.58$^{a***}$ | 14.9±0.89$^{a***}$ |
| Standard | 13.0±1.00$^{b***}$ | 28.0±2.55$^{b***}$ | 29.1±0.74$^{b***}$ |
| Low Dose | 35.2±2.28$^{c***}$ | 24.0±1.22$^{c***}$ | 20.2±1.27$^{c**}$ |
| High dose | 45.8±2.58$^{d***}$ | 30.6±1.14$^{d***}$ | 29.2±1.92$^{d***}$ |

Control: CMC; PCOS control: Letrozole; Standard: Clomiphene citrate; Low dose: Extract 100 mg/kg; High dose: Extract 200 mg/kg; $^{a}$PCOS control vs. control; $^{b}$Standard vs. PCOS control; $^{c}$Extract low dose vs. PCOS control; $^{d}$Extract high dose vs. PCOS control; *P < 0.05, **P < 0.01, ***P = 0.001, $^{ns}$not significant, n = 6.

**Table 3: Effect of various treatments on lipid profile.**

| Group | TG (mg/dl) | Cholesterol (mg/ml) | HDL (mg/ml) | LDL (mg/ml) |
|---|---|---|---|---|
| Control | 82.4±2.96 | 81.2±3.96 | 37.8±3.11 | 26.8±1.92 |
| PCOS Control | 139±1.48$^{a***}$ | 132±6.87 $^{a***}$ | 19.0±1.58 $^{a***}$ | 85.4±2.07 $^{a***}$ |
| Standard | 63.6±5.03$^{b***}$ | 75.8±8.10 $^{b***}$ | 27.8±2.58 $^{b***}$ | 39.0±1.58 $^{b***}$ |
| Low Dose | 85.4±18.28$^{c***}$ | 86.0±5.70 $^{c***}$ | 25.4±4.03 $^{c**}$ | 35.2±1.30 $^{c***}$ |
| High dose | 62.2±3.96$^{d***}$ | 70.2±3.56 $^{d***}$ | 33.6±1.67 $^{d***}$ | 20.6±5.22 $^{d***}$ |

Control: CMC; PCOS control: Letrozole; Standard: Clomiphene citrate; Low dose: Extract 100 mg/kg; High dose: Extract 200 mg/kg; $^{a}$PCOS control vs. control; $^{b}$Standard vs. PCOS

control; [c]Extract low dose vs. PCOS control; [d]Extract high dose vs. PCOS control; *P < 0.05, **P < 0.01, ***P = 0.001, [ns]not significant, n = 6.

**Table 4: Effect of various treatments on antioxidant enzymes.**

| Group | SOD(units/mg protein) | Catalase (µmole of $H_2O_2$ consumed per mg protein) |
|---|---|---|
| Control | 66.2±2.00 | 3.0±0.28 |
| PCOS Control | 36.0±2.91[a***] | 0.2±0.05 [a***] |
| Standard | 39.8±1.64[b*] | 2.4±0.27[b***] |
| Low Dose | 73.4±2.88[c***] | 2.6±0.16[c***] |
| High dose | 83.4±2.30[d***] | 3.5±0.15[d***] |

Control: CMC; PCOS control: Letrozole; Standard: Clomiphene citrate; Low dose: Extract 100 mg/kg; High dose: Extract 200 mg/kg; [a]PCOS control vs. control; [b]Standard vs. PCOS control; [c]Extract low dose vs. PCOS control; [d]Extract high dose vs. PCOS control; *P < 0.05, **P < 0.01, ***P = 0.001, [ns]not significant, n = 6.

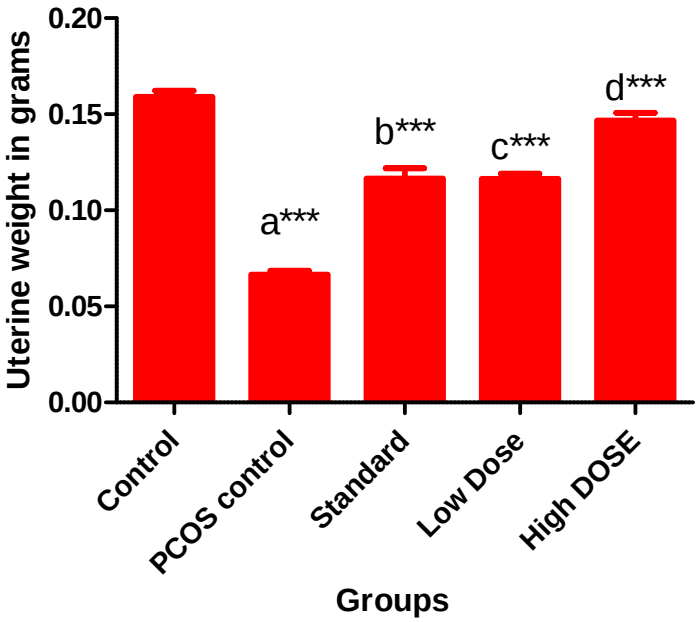


**Figure 1: Effect of various treatments on uterine weights.**

**a- PCOS control vs control**

**c- Extract Low Dose vs PCOS control**

**d- Extract High Dose vs PCOS control**

***→ *P*<0.05, **→ *P*<0.01, ***→ *P*<0.001**

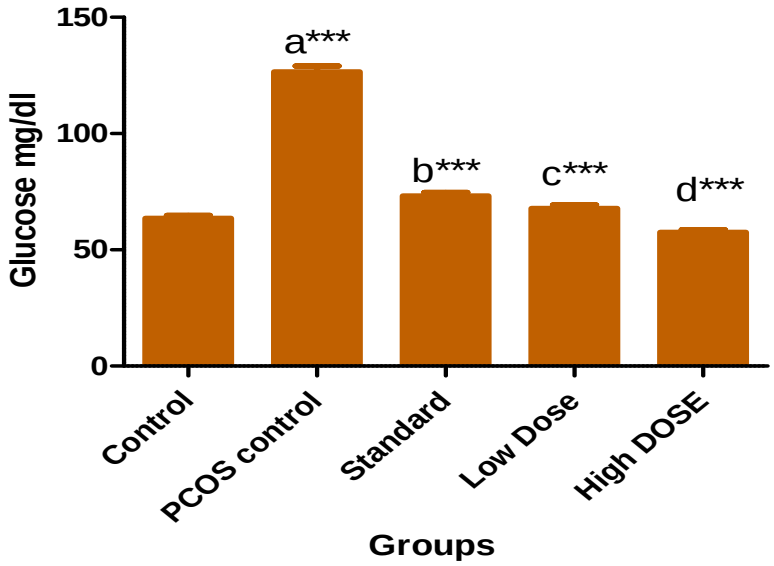


**Figure 2: Effect of various treatments on serum Fasting Blood Glucose levels.**

**a- PCOS control vs control**

**c-Extract Low Dose vs PCOS control**

**d- Extract High Dose vs PCOS control**

***→ *P*<0.05, **→ *P*<0.01, ***→ *P*<0.001**

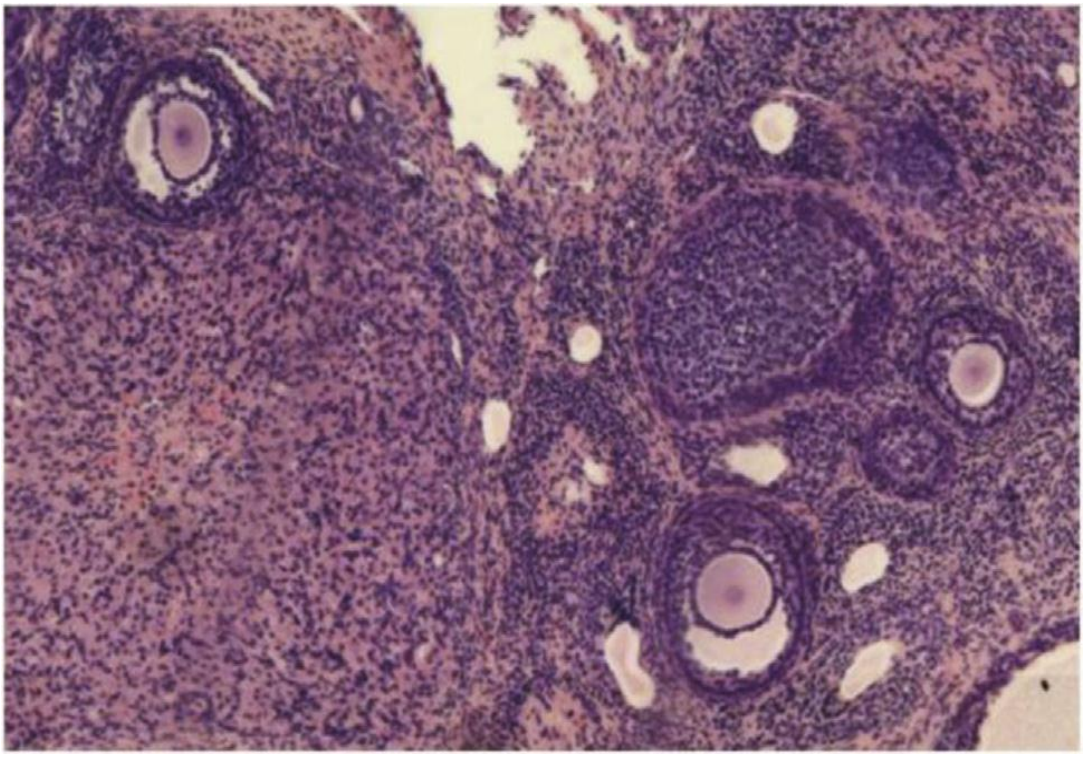

**Figure 3: Section of ovary from control animal showing follicles (H&E,X10).**

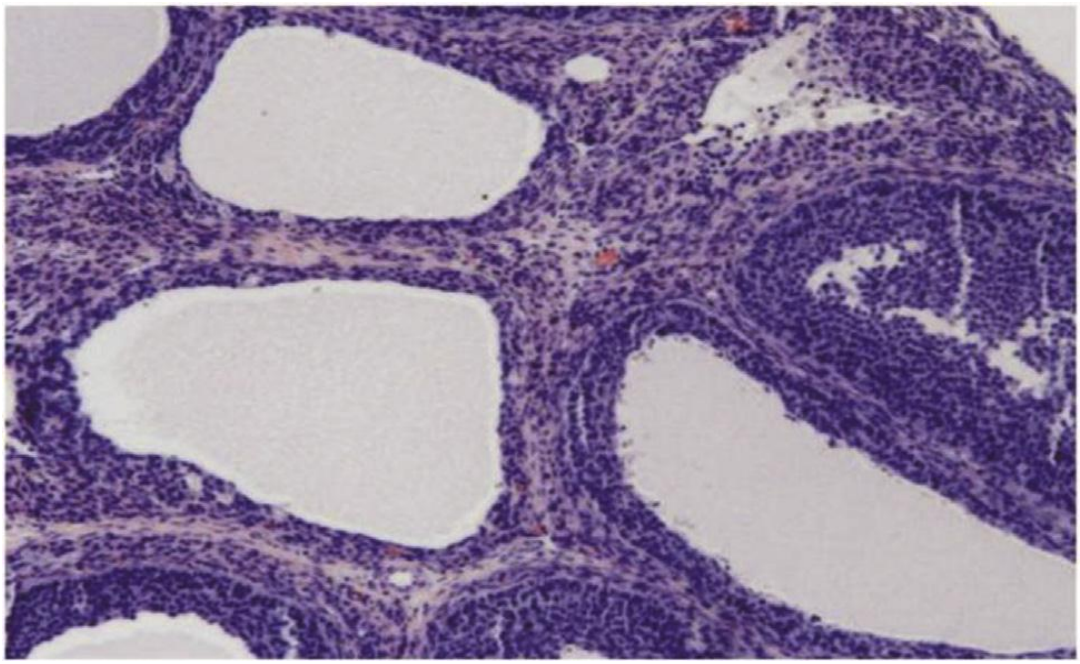

**Figure 4: Section of ovary from PCOS induced animal showing Multiple cysts. (H&E,X10).**

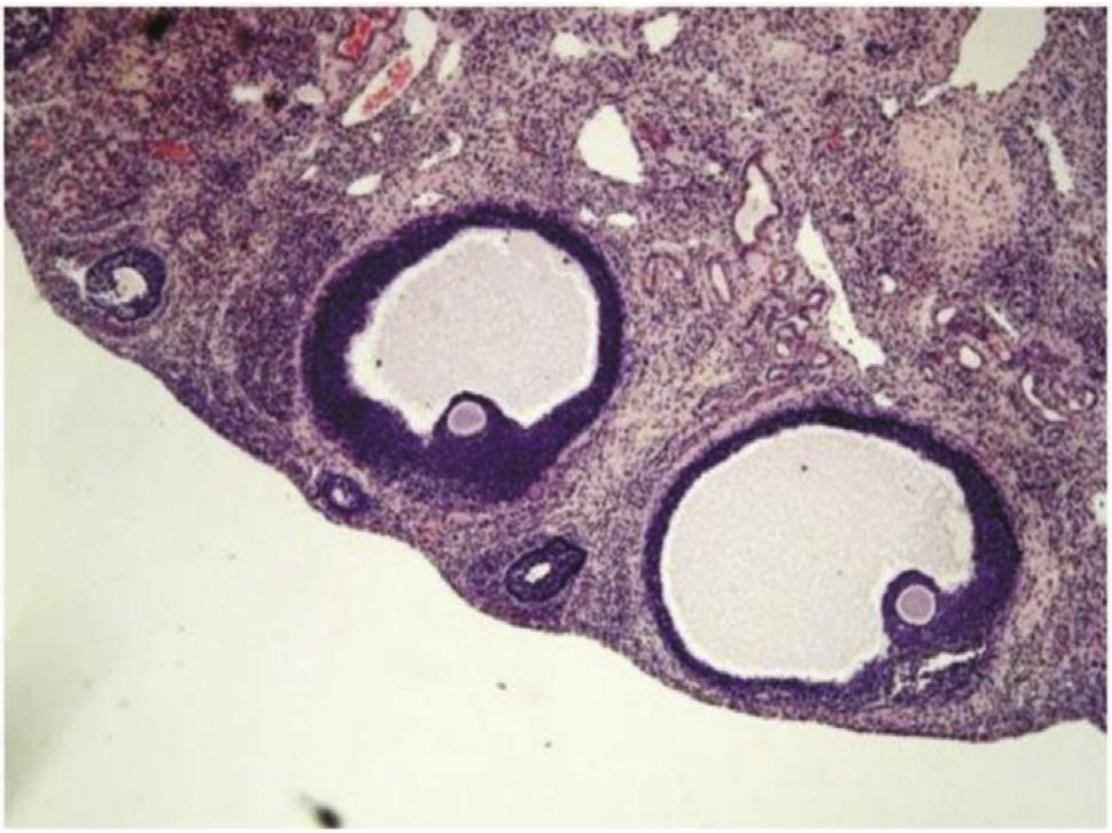

**Figure 5: Section of ovary from Extract treated (100 mg/kg) animal showing large follicles. (H&E, X5).**

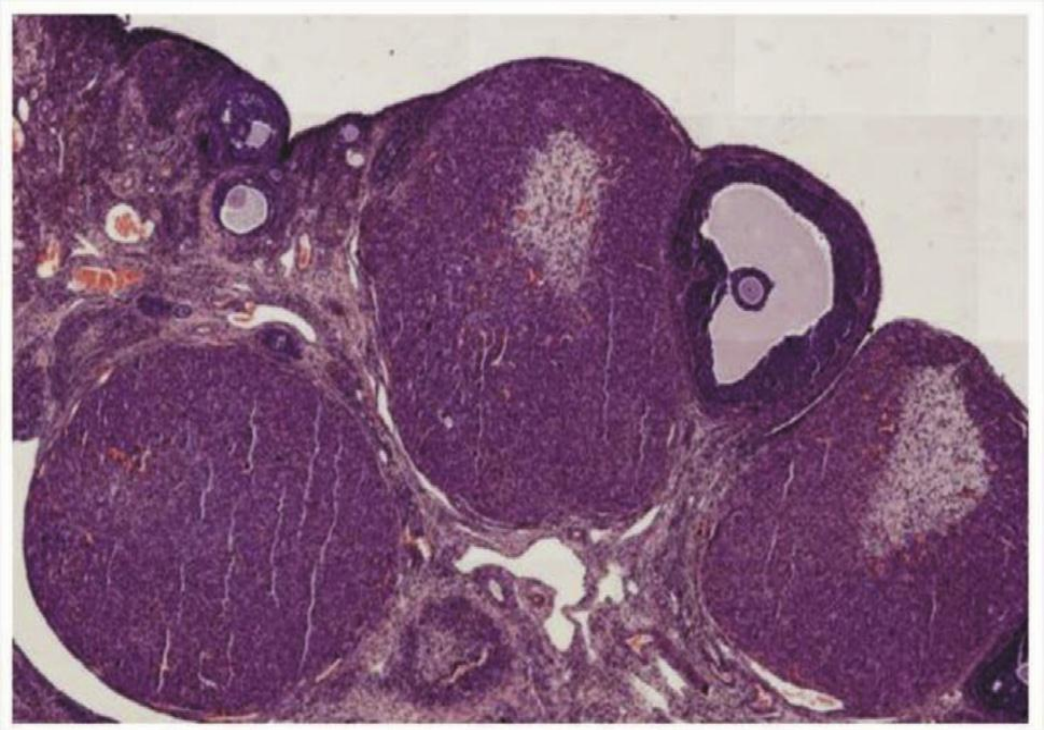

**Figure 6: Section of ovary from Extract treated (200 mg/kg) animal showing follicles at different developmental stages and corpora lutea. (H&E, X10).**

## 4. DISCUSSION

In the present study, Letrozole-aromatase inhibitor, was used to induce Polycystic Ovary Syndrome in female Wistar rats. Previous reports suggest that Letrozole induced PCOS condition depicts human PCOS in many ways.[6] The working of this model was confirmed by regular examination of vaginal smears and presence of persistent vaginal cornification. As evidenced, there was a marked increase in testosterone levels when compared to control animals indicating the hyperandrogenism status in PCOS condition.

Extract was able to normalize serum Testosterone levels similar to that of Clomiphene citrate. Serum levels of Progesterone and Estradiol were decreased in PCOS induced group. These results are in accordance with the earlier studies.[12] Decreased progesterone levels are also indicative of anovulation[11] and extract successfully restore its level to normal. Decreased estradiol concentration due to inhibition of aromatase in PCOS induced group was significantly increased by repetitive administration of high dose (200 mg/kg) of extract, confirming its earlier reported phytoestrogenic activity.[3]

Even though, there were no significant changes in ovarian weights, uterine weights were reduced due to Letrozole treatment.[6,5] Extract treatment significantly increased the uterine weights which matched to those in control animals.

One of the consequences of PCOS is dyslipidemia. Imbalances in lipid profile are attributed to hyperandrogenemia.[12] Present study exhibited similar results in lipid profile. PCOS induced group showed notable increase in TC, TG's, LDL and decrease in HDL levels. Extract displayed its antihyperlipidemic action by considerably decreasing serum TC, TG's, LDL while increasing HDL levels.

Many studies reported oxidative stress as one of the pathological factor for PCOS.[7,9] Increased oxidant levels may alter the stereo diagnosis in ovaries contributing to increased androgen production and polycystic ovaries.[9] In the present study, it was observed that the PCOS animals exhibited elevated oxidative stress markers and reduced endogenous antioxidants in ovary. SOD and Catalase activity were significantly diminished in the PCOS group and concomitant treatment with extract restored their activities.

Extract treatment advanced to disappearance of cysts and decreased incidence of pyknotic granulosa cells. Varying number of corpora lutea were seen suggesting ovulation and normal estrous cyclicity. Follicles at different stages of development with oocytes and clear, visible granulosa cell layer were observed. Ovarian cortex appeared normal with many follicles.

## 5. CONCLUSION

Ethanol leaf extract of OS showed many beneficial effects similar to Clomiphene citrate in treating PCOS condition and inducing ovulation. It restored the hormone and lipid profile, antioxidant and glycemic status as well as ovarian morphology in Letrozole induced PCOS animals. These effects may be ascribed to its multiple pharmacological activities like estrogenic, antihyperlipidemic, antioxidant and hypoglycemic effects which could be useful in managing PCOS condition and prevent ovarian cell dysfunction, ovulation and thereby improving fertility. Together broad spectrum biological effects of this Extract make it a promising drug for treating clinical and pathological abnormalities in PCOS condition.

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