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Research Article

Beneficial Effects of *Prunus dulcis* (Almond Oil) on Letrozole-Induced Polycystic Ovarian Syndrome in Rats

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ABSTRACT

Prunus dulcis (Almonds) are cholesterol free and contain many vital health nutrients like zinc and L-arginine, and have high amount of monounsaturated fats (MUFA's) required for reproductive health. PCOS is a recurrent endocrine disorder in females, and it affects around 6%-10% of all female population. Letrozole is a drug hypothesized to induce polycystic ovarian syndrome (PCO) in experimental rat models. The goal of this study was to analyze antiandrogenic effects of Almond oil on PCOS which is produced by letrozole in rats. Animals were separated into three different groups (n=6). Control (untreated) group was considered as group I. PCOS was induced in rats with 1mg/kg of Letrozole daily for 21 consecutive days in Group II, while Group III was treated with letrozole for 21 days along with Almond oil (0.1ml/kg b.w) daily for last 14 consecutive days of the experiment. Body weights were recorded and vaginal smears were prepared and fixed to deduce the phases of estrus cycle on daily basis. 21 days later rats were slaughtered and the ovaries were collected for histological investigations and blood plasma was stored for further biochemical assessments. There are four phases in Estrus cycle: estrus, metestrus, diestrus and proestrus. Group II, the cycle was blocked by letrozole at diestrus phase as shown by vaginal smear. After almond oil administration Group III showed some positive response and the diestrus phase preceded to estrus phase. Weight loss was observed in both Groups I & II. Our biochemical test exhibited some positive results as the testosterone, and triglycerides decreased and even the cholesterol profile, but only HDL were slightly increased in the treated group. Almond oil's prolonged usage may exert some beneficial effects in PCOS. Treatment with this oil might gradually reverse the diestrus phase into normal estrus phase. It is also able to decrease the hyper androgenic condition by decreasing testosterone levels and hyperglycemic condition changed to normoglycemic due to MUFA's and phenolic compounds found in almond oil.

Key words: *Polycystic ovarian syndrome, Letrozole, almond oil, Estrus cycle, Antiandrogenic, Vaginal smears.*

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is the recurrent endocrine disorder in females, and it is affecting around 6%-10% of all female population[1]. Stein and Leventhal the two American gynecologist who were the first to discover about this disorder[2].

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An Italian medical scientist and physician Vallisneri in 1721 reported that an infertile woman with shiny ovaries, white surface and size of pigeon eggs is suffering from PCOS[3]. This disorder involves multiple organ systems within the body since it affects most of the hormones and the major underlying cause of the syndrome is due to the insensitivity of insulin. Some of the females who are suffering with PCOS can rectify for their insulin resistance (IR), yet an enormous proportion of them have altered beta-cell function[4] causing glucose intolerance, which increases the risk of developing type 2 diabetes (T2D), independently of age and body mass index (BMI)[4]. PCOS is also sometimes known for the hyper-androgenic conditions, which is correlated with oligo-anovulation and ovarian morphology defects[5,6]. Androgen secretion is enhanced in most of the women with PCOS who are either overweight or obese, which compromises the metabolism and reproductive functions it also favors the PCOS phenotype development[7]. Psychological impairments, including depression, mood disorders and metabolic disturbances are incorporated with the manifestations of the disorder[8,9]. However, PCOS is one leading cause of sterility, and all of the other reproductive characteristics are secondary[10]. Gestational diabetes, preeclampsia and miscarriages are outcomes of anovulatory infertility which is there during the fertile period[11]. Close follow-up of these symptoms and early diagnosis is highly significant to reduce the risk of further complications[10]. Gestational diabetes, preeclampsia and miscarriages are outcomes of anovulatory infertility which is there during the fertile period[11]. Despite the fact that there is lack of criteria for the diagnosis of PCOS and the etiologies of this disorder is not completely understood but still there might be ideologies for it. A strong correlation is there between hyperinsulinemia and hyperandrogenism (HA) in PCOS, but the association behind it are still not clear[12].

In South Asian women, predominantly in Pakistani women the prevalence of PCOS is much higher (52%) as compared to other population such as White people (20 - 25% in UK). Local researches that can give an insight into the primordial factors causing the syndrome is scarce[13]. Pathophysiology of PCOS is quite complicated and most of it is not clear. Though hormonal imbalance created by a combination of increased androgens and insulin resistance might be the major cause of it[14]. Genetic and environmental factors promotes the hormonal disturbances in combination with other determinants, such as obesity, fetal reprogramming hypothalamic-pituitary abnormalities (HPA) and ovarian dysfunction which contributes to the causes of PCO[14,15]. Pathophysiological contributors have been hindered due to lack of exemplary methods to assess either the aetiology belongs to hyperandrogenism or insulin resistance. Most of the studies suggest that a deep rooted contributor of PCOS is hyperandrogenism, and around 60% to 80% of cases have been reported. Insulin resistance is also a pathophysiological contributor, which affects around 40% to 60% of women suffering from PCOS[16] and it contributes not only to metabolic features but also to reproductive features as it enhances the androgen production by reducing the SHBG (hormone binding globulin) and intensifying free androgens in the body[8].

Women suffering from PCOS may exhibit a few serious clinical consequences which includes psychological problems such as mood disorders, depression, anxiety, decreased quality of life and self-esteem[17,18], some reproductive manifestations which are mostly associated with hyperandrogenism are hirsutism, acne, and infertility[19]. Insulin resistance, metabolic syndrome, type II diabetes mellitus (DM2) and high possibility of cardiovascular diseases are few other implications of PCOS[4,20]. PCOS is also manifested by amenorrhea, anovulatory cycles, menstrual irregularities, alopecia and acanthosis nigricans occasionally but not constantly in relationship with proliferated cystic ovaries[21].

Polycystic ovaries can be instigated by androgen exposure and it not only includes the

exogenous androgens but it is also an outcome of secondary endogenous androgen excess[22,23]. The latter consists of the experimental model for PCOS, letrozole was induced in rats which is a nonsteroidal aromatase inhibitor and it blocks the conversion of androgens to estrogen[24]. Letrozole treatment initiates acyclicity in adult rats when treatment is given for at least 21 consecutive days or more than that[25]. Large follicular cysts may appear in ovaries with reduced or no corpora lutea with irregular estrus cycle and anovulation[27].

Monounsaturated and Polyunsaturated fatty acids (MUFA and PUFA) are two major components of nuts which benefits the metabolic implications associated to PCOS. The MUFA and PUFA elevate plasma lipids, and PUFA (particularly n-3) amplifies insulin sensitivity, it even decreases blood pressure and inflammation[26-28]. As there is a high concentration of MUFA and PUFA in nuts it is recommended for PCOS patients to increase nut intake. Almond is classified as a nut by different regions, but it is actually the seed (drupe) from the fruit of an almond tree[29]. They are a rich source of protein, vitamin B2 (riboflavin) and vitamin E, with that it even has 30% MUFA and 12% PUFA content. Almonds are rich in essential reproductive system nutrients including zinc and L-arginine and are thought to have the most of fertility nutrients. In women, vitamin E may assist in regulating the production of cervical mucus, which is important for conception, due to its capability to support live sperm for several days. Almonds also help in improving insulin resistance, a condition where the body becomes less potent at lowering blood sugar levels. Insulin resistance can decrease fertility in women, especially when combined with abdominal weight gain[30].

However, the goal of the study was to ponder at the anti-androgenic effects of almond oil on letrozole induced PCOS in rats. As almond can be easily available and are economically feasible for all the female who are struggling with PCOS.

In conclusion, the results based on the biochemical assessment, histological findings and vaginal smears in this study demonstrates that prolonged use of almond oil can have beneficial effects in PCOS phenotypes by producing an antiandrogenic effect

MATERIAL AND METHODS

2.1 EXPERIMENTAL PROTOCOL

2.1.1 SELECTION OF ANIMALS:

9-12 weeks old, 200-220g female Wistar rats were chosen for this study and acquired from DOW University of health sciences, Karachi.

2.1.2 HOUSING AND DIET:

Animals were acclimatized in the animal house of Department of Physiology (University of Karachi). The animals were grouped according to their weights (n=6) and kept in a room under normal regulated temperature (22- 24 C), and in a reversed 12hr light-dark cycle. Free access was provided for water and diet prepared according to their daily requirements and was estrogen free diet. All the experiments performed in this study were based on the recommendations given by Institutional Animal ethical Committee.

2.2 EXTRACTION OF PRUNUS DULCIS (ALMOND OIL):

Almonds were purchased from a local market in Karachi. Almond oil was extracted by three-phase partitioning method in which specified amounts of ammonium sulphate and t-butanol are added to the aqueous suspension of the almond[31,32] . At first, almond seeds were de-shelled, as the nut was obtained it were then soaked in water for 1h to peel the skin and then grounded to form powder[33]. In a magnetic stirrer slurry was made by dissolving almond powder in distilled water (5g/40 mL, pH 4.0 in almond) and gentle stirring. Then it was time to add required amount of ammonium sulphate and t-butanol and swirled gently. For the three-phase formation slurry was brooded for 1 hour at optimum temperature. Later on, it was separated by centrifugation at 2000 × g for 10-15 min. The upper layer of the suspension was extracted and the final step of evaporation took place on a rotary evaporator (under vacuum for 5 min at 50℃) to obtain oil distillated in this phase and then the almond oil was used in our experiment[33].

2.3 STUDY DESIGN:

Animals were partitioned into three different groups and each group comprised of six female rats. Estrous cycle is of 4 days and the experimental rats had normal estrous cycle. After keeping them under observation for a day, from the next day the doses were then being administered orally.

GROUP I: untreated (control) group, animals were given proper diet and water.

GROUP II: letrozole treated group, Letrozole (1mg/body weight) was given to rats for 21 consecutive days.

GROUP III: was treated with letrozole for 21 days along with Almond oil (1 ml/kg b.w) daily for 14 consecutive days.

2.4 PCOS INDUCTION:

Experimental animals were treated with Letrozole (Lezra 2.5mg manufactured by Excel Health Care) except control group. In 0.9% NaCl ,1mg/body weight letrozole was dissolved and was orally administered to the animals with the help of gavage, and it was given once daily for 21 consecutive days.

2.5 VAGINAL SMEARS:

Estrus cycles were interpreted by microscopic evaluation of the predominant cell type in vaginal smears which were acquired daily for 21 consecutive days till the animals were sacrificed. Animals that were treated with letrozole were arrested in the pseudo-diestrus phase when vaginal smears exhibited leukocytes as the dominant cell type.

2.6 SLIDE PREPARATION & FIXATION

To assess the phase of the estrous cycle visually, all the rats were grasped by its tail and turned back to make the vaginal smear. The vaginal smear was collected by utilizing cotton tipped swab which was wetted in medium temperature physiological saline, and later on it was inserted in the vaginal opening of the hampered mouse. The swab was delicately rolled against the vaginal wall and then withdrawn. By gently rolling the swab along the slide, cells were moved to the slide.

All of the slides were then either air dried or fixed under slight flame and then stained with around 400µl of crystal violet stain. The slides were dipped in the crystal violet solution for 30 seconds and after that they were rinsed with water and again air dried. They were immediately observed and captured at the magnification of 10X and 40X under the bright filed illumination. This process was repeated for 21 consecutive days.

2.7 OVARIAN TISSUE COLLECTION

2.7.1 BIOCHEMICAL ANALYSIS:

All of the rats were anesthetized and were kept for eradication by rapid heart blood sampling method. In this method blood was taken out immediately from the heart while it was still pumping.About 4-5ml blood was taken out and was centrifuged for 15-20min, 2500r/min. The upper layer of plasma was collected in micropippete and then shifted into Eppendorf tube, and then preserved at -86℃ for further analysis.

2.7.1.1 MEASUREMENT OF GLUCOSE:

Glucose test was performed by GOD-PAP method, a commercial diagnostic kit was used[34,35].

2.7.1.2 ASSESSMENT OF LIPID PROFILES:

Lipid profile [total-cholesterol (TC)[36], triglycerides (TG)[37], and HDL-cholesterol (HDL-C)[38] were evaluated by using enzymatic endpoint test. LDL-C was figured out by using the Friedewald's equation.

2.7.1.3MEASUREMENT OF TESTOSTERONE:

Testosterone is measured by the ELISA kit by using the plasma of rats. ELISA is the enzyme-linked immunosorbent assay and it works on solid phase competitive binding[39].

2.7.2HISTOPATHOLOGICAL EXAMINATION:

Ovaries were secured in 10% buffered neutral formalin and then converted into parraffin imbedded blocks. By using microtome, 5µm thick sections were made from the blocks, and then implanted on poly-lysine coated slides. After few minutes it is stained with eosin and hematoxylin.Slides were kept under light microscope which was connected to a camera to observe and capture images of ovaries[40].

Scoring:

Score 0= Absent (0%)

Score 1= Mild (+)(10-20%)

Score 2= Moderate (++)(30-50%)

Score 3= Severe(+++)(>50%)

2.8 STASTICAL ANALYSIS:

The data collected is represented as mean ± standard deviation. Stastical analysis was completed by SPSS version 16. The data was statistically analyzed by independent T-test. ρ values which are lesser than 0.05 were reported as significant.

RESULTS

3.1ESTROUS CYCLE DETERMINATION THROUGH VAGINAL SMEARS:

Estrous cycle is the reproductive cycle in rats of 4-5 days and it comprises of four different phases. Vaginal smear is distributed into 4 phases of the cycle which are known as: Estrus (E), Diestrus (D), Proestrus (P), and Metestrus (M)[41]. Briefly, 3 major types of cells could be identified:

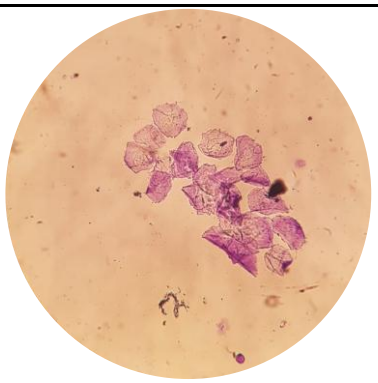
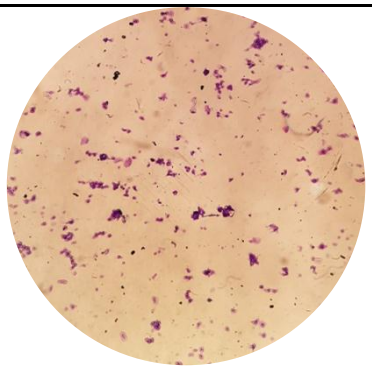
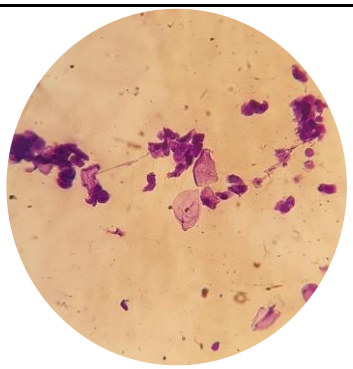
(i) Epithelial cells which are round and nucleated,

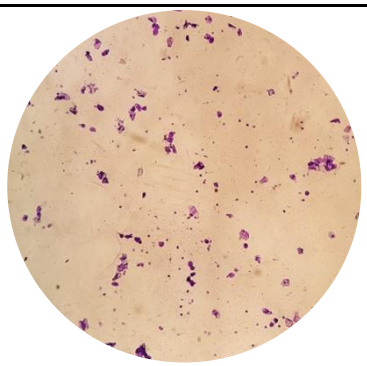
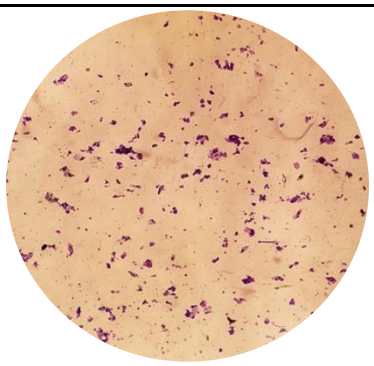
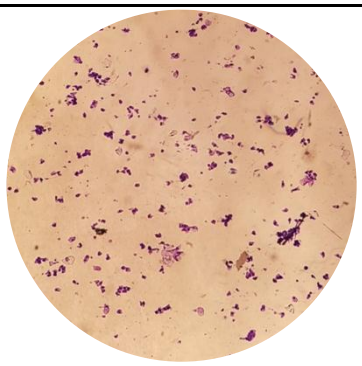
(ii) Cornified cells which are irregular and without nucleated,

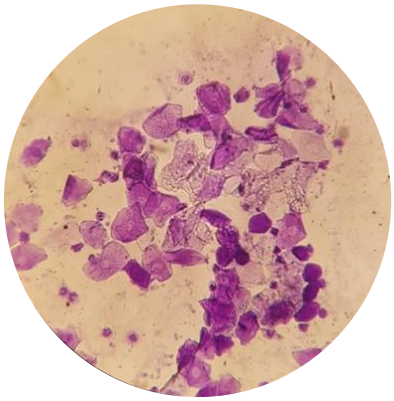
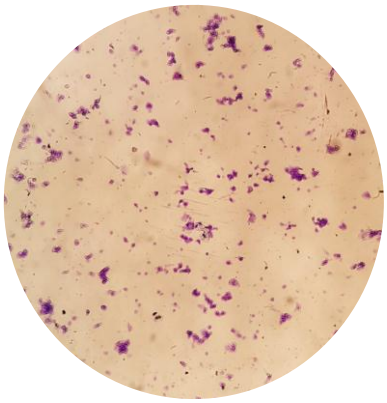
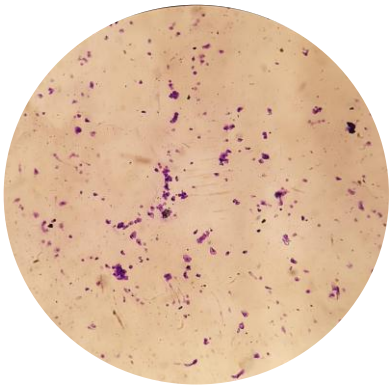
(iii) Leukocytes are round and small. .

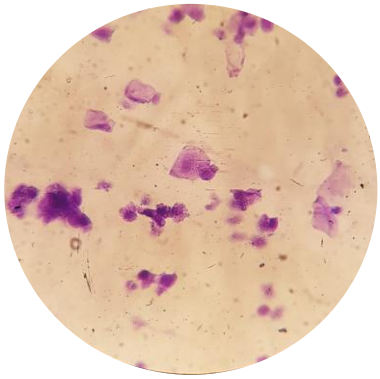
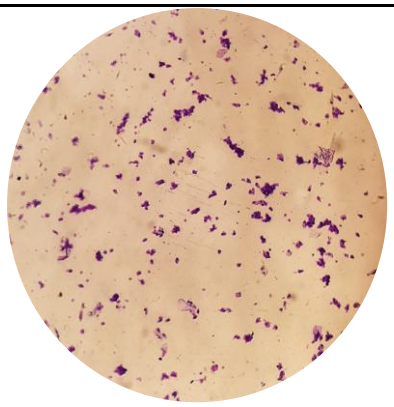
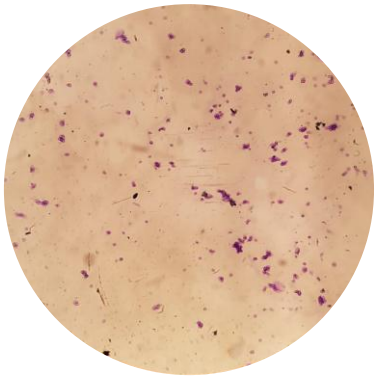
The contrasting amount of cells visualized in the smears was used for determining the phase of estrous cycle. When dominance of epithelial cells is seen in the smear it is proestrus phase. Smear which mainly consists of cornified cells is known as estrus phase. When same proportion of cornified, leukocytes and epithelial cells are observed it was considered as metestrus phase. Lastly, the smear which has leukocytes is considered as diestrus phase[42].

Table 1: The vaginal smear of rats with various stages of estrus cycle in the untreated control group, letrozole treated and letrozole + almond treated at magnification of 40X.

DAY 1		
CONTROL GROUP	**LETROZOLE TREATED**	**LETROZOLE + ALMOND TREATED**
		
ESTRUS	**DIESTRUS**	**PROESTRUS**

DAY 6		
CONTROL GROUP	**LETROZOLE TREATED**	**LETROZOLE + ALMOND TREATED**
 **DIESTRUS**	 **DIESTRUS**	 **DIESTRUS**

DAY 15		
CONTROL GROUP	**LETROZOLE TREATED**	**LETROZOLE + ALMOND TREATED**
 **DIESTRUS**	 **DIESTRUS**	 **DIESTRUS**

DAY 21		
CONTROL GROUP	**LETROZOLE TREATED**	**LETROZOLE + ALMOND TREATED**
		
METESTRUS	**DIESTRUS**	**DIESTRUS**

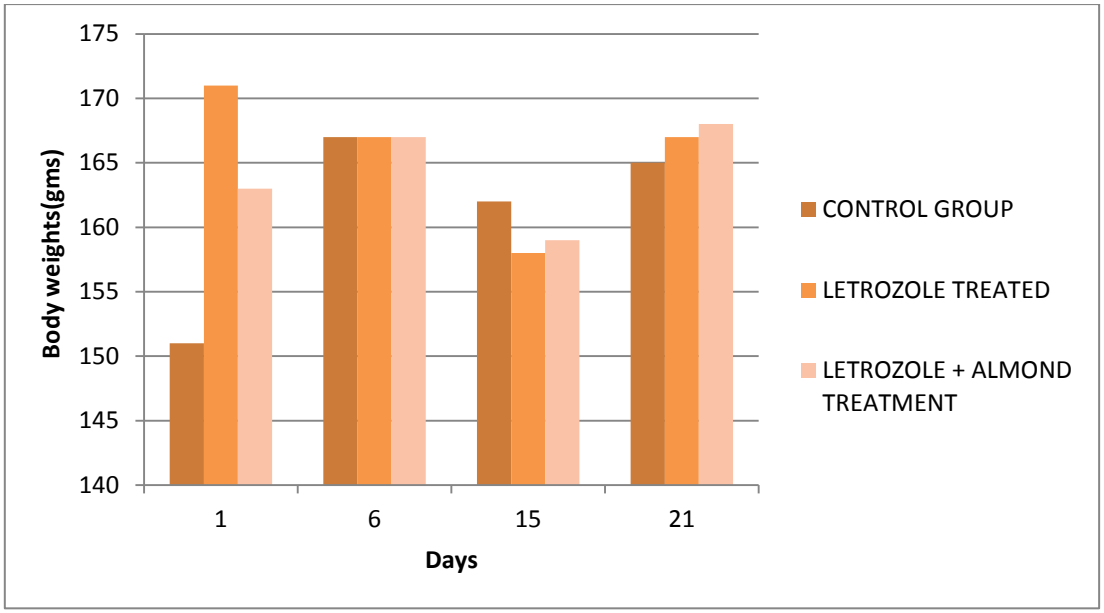


Fig. 1: Effect of almond oil treatment in letrozole treated group on weekly body weight measurement

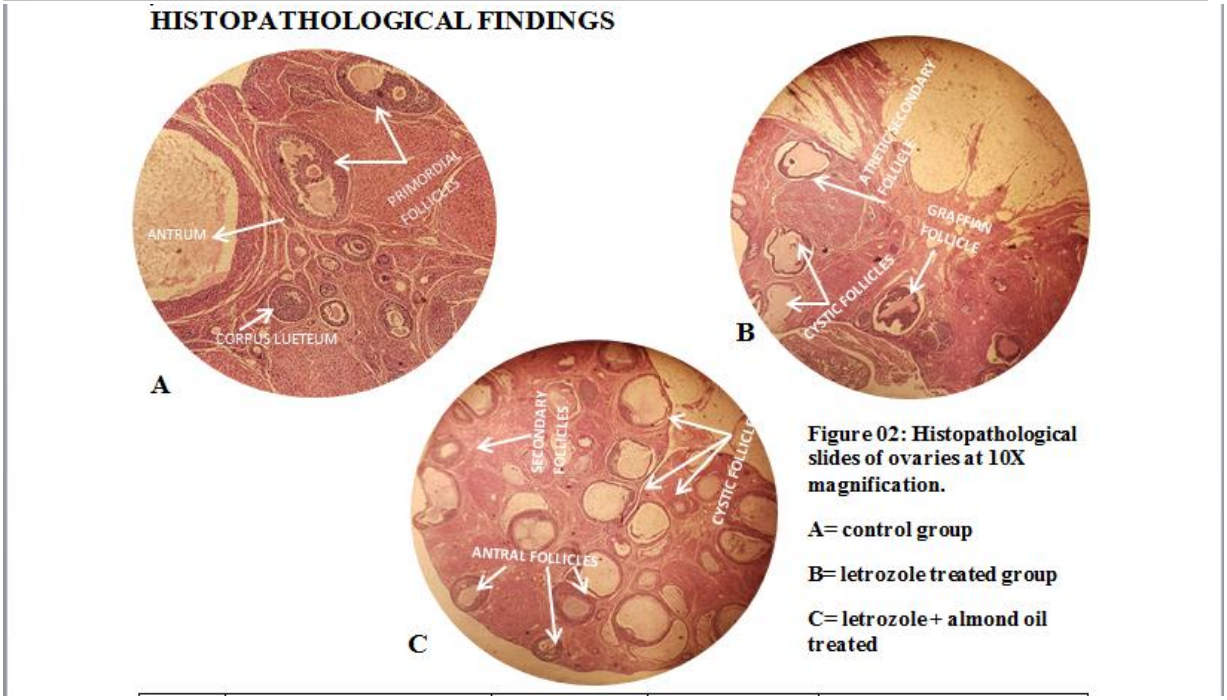


Figure 02: Histopathological slides of ovaries at 10X magnification.

A= control group

B= letrozole treated group

C= letrozole+ almond oil treated

S.NO	HISTOLOGICAL FEATURES	CONTROL	LETROZOLE TREATED	LETROZOLE + ALMOND OIL TREATED
1	HYPERPLASIA OF THECA INTERNA	+	+	+
2	CAPSULAR THICKENING	0	0	0
3	ATROPHY OF CORPORA LUTEA	0	0	0
4	HYPERTROHY OF CORPORA LUTEA	None	++	++
5	ATRETIC PRIMARY FOLLICLE	Occasional	Occasional	Occasional
6	ATRETIC SECONDARY FOLLICLE	Few	Many	Many
7	ATRETIC GRAFFIAN FOLLICLE	0	0	0
8	GRANULOSA LUTEINIZATION	+	+	+
9	ENCAPSULATED GRANULOSA	0	0	0
10	NUMBER OF CYST	0	0	0
11	NUMBER OF FOLLICLES	32	16	35
12	FOLLICLE SIZE (in mm)	Variable 1mm	Variable 2mm	Variable 2mm

Table 2: Histopathological features of ovaries in control group, letrozole treated and letrozole + almond oil treated.

Scoring: absent (0), mild (+), moderate (++), severe (+++)

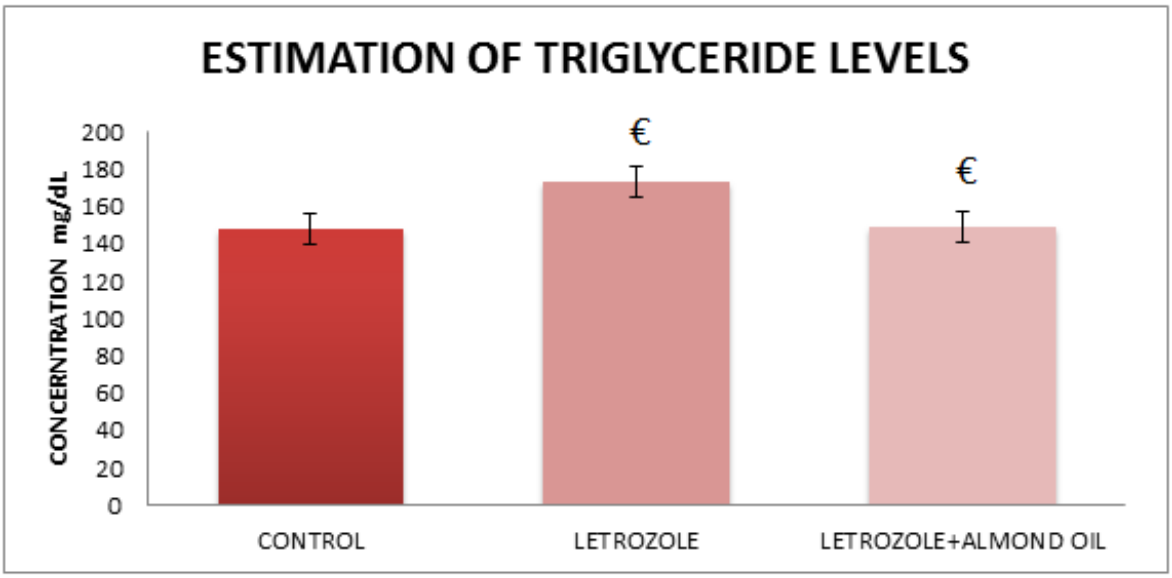
Absent 0%, mild 10-20%, moderate 30-50%, severe > 50%.

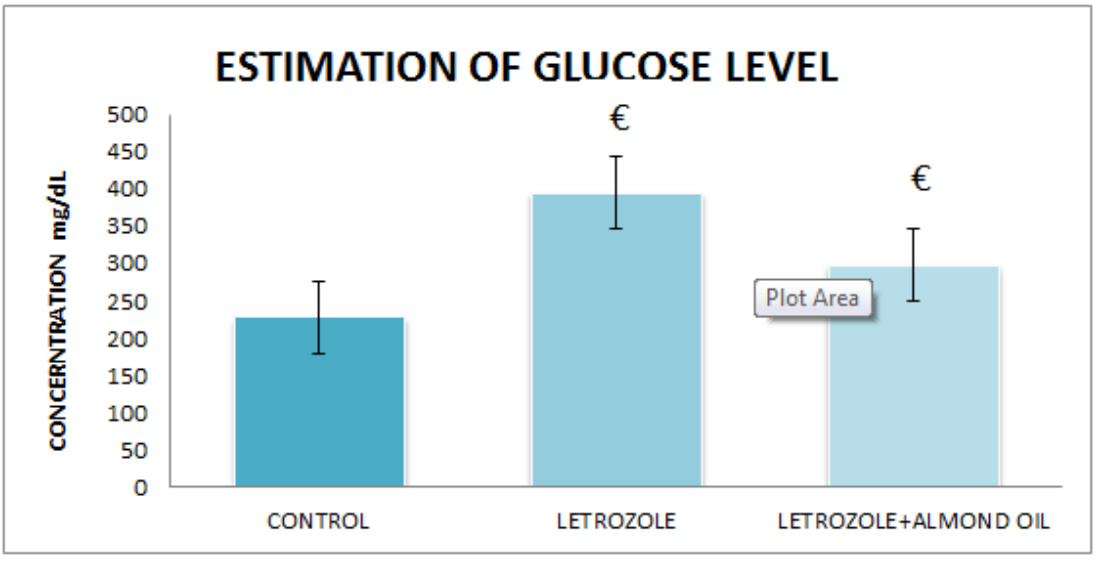
BIOCHEMICAL ASSESSMENT

PARAMETERS	CONTROL	LETROZOLE TREATED	LETROZOLE + ALMOND OIL TREATED
GLUCOSE (mg/dL)	227.79 ± 6.59	395.31 ± 4.17€	297.16 ± 19.07€
TRIGLYCERIDE (mg/dL)	147.73 ± 4.96	173.27 ± 2.15€	149.286 ± 5.32€
CHOLESTEROL (mg/dL)	137.53 ± 2.46	197.56 ± 0.639€	113 ± 3.55€
HDL (mg/dL)	56.05 ± 0.63	53.35 ± 0.88¥	47.83 ± 1.28€
LDL (mg/dL)	42.82 ± 2.39	113.82 ± 3.15€	30.64 ± 0.50€
TESTOSTERONE (ng/dL)	0.789 ± 0.09	4.05 ± 0.35€	1.45 ± 0.18€

Table 3: Effect of almond oil on serum lipid profile, testosterone and glucose intolerance in control group, letrozole treated and letrozole + almond oil treated

Values are commuted as mean ± Standard deviation, n=6 animals in each group. Symbols represent stastical significant €: p < 0.05 and non – significant ¥: p > 0.05.





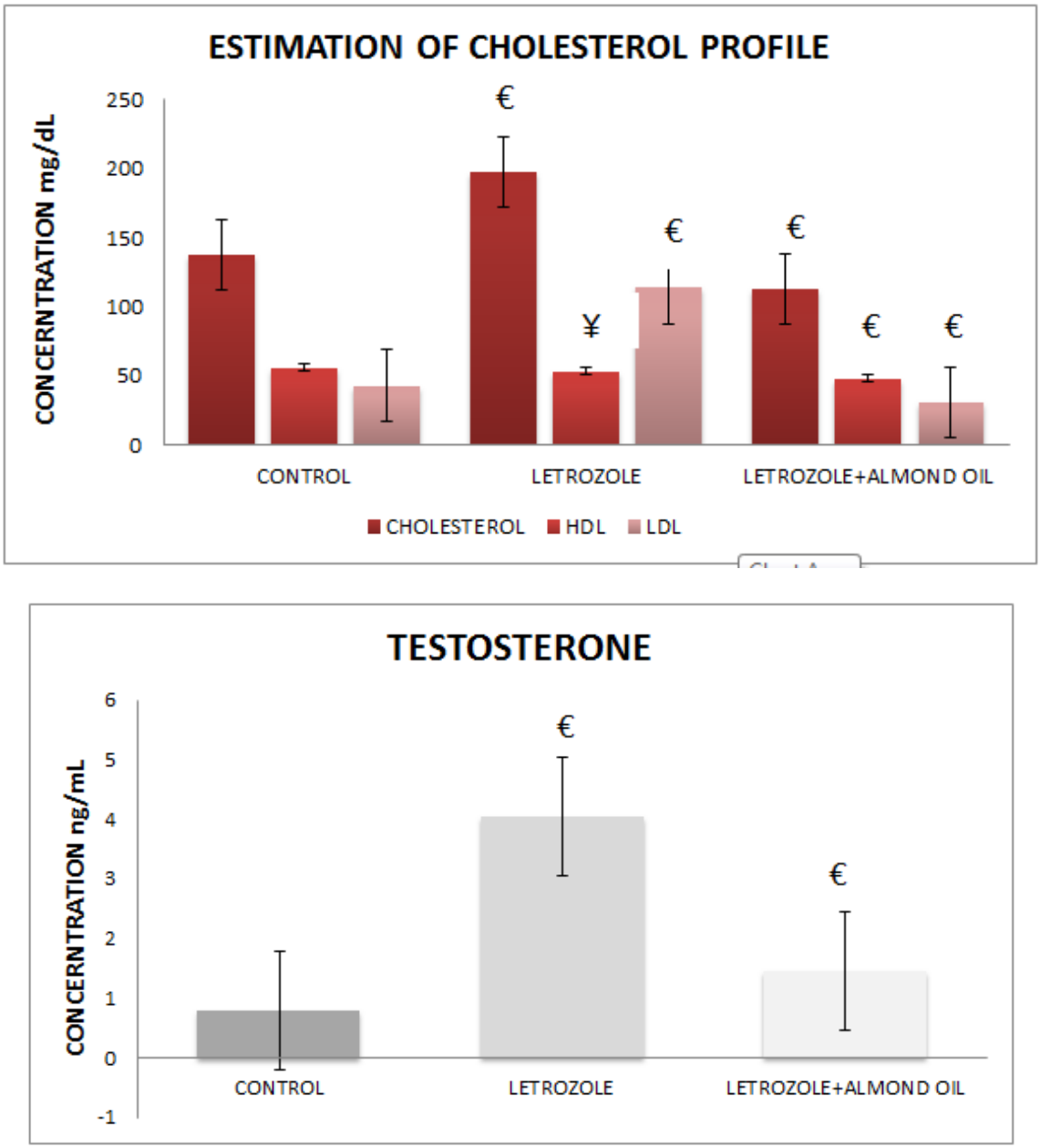


Fig. 4: Effects of almond oil on control, letrozole treated and letrozole + almond oil treated. €: ($p < 0.05$) significant, ¥: ($p > 0.05$) non-significant

DISCUSSION

Polycystic ovarian syndrome has many manifestations, which includes oligomenorrhea, insulin resistance and hyper-androgenism, and may lead to metabolic dysfunction[43]. In this present study, the biochemical assays and clinical attributes of PCOS in a rat model were analyzed. Letrozole is an aromatase inhibitor which is induced, hence it increases ovarian androgens and androgen substrates are not converted into estrogens as the pathway is blocked[44]. It would lead to hyperandrogenism, a signifying attribute of PCOS[45].

Previous studies suggests that letrozole induced PCOS condition depicts human PCOS in many ways[24]. In the current study, the untreated control group rats showed a regular estrous cycle. After a few days, regular estrus cycle was disturbed in letrozole treated group and it exhibited a persistent diestrus phase. The working of this model was supported by regular examination of vaginal smears and presence of persistent vaginal cornification. Treatment of almond oil slowly reversed the diestrus phase to a normal estrous phase on the last day of experiment. The changes in the estrous cycle of rats might be connected to

alterations in the circulating concentrations of the sex hormones, as these hormones regulate the ovarian function, including follicular maturation and hormonal imbalances. It is possible that all of these factors have led to irregular estrous cycle and anovulation[41].

PCOS is also known as metabolic disorder related with T2diabetes mellitus and manifests as hyperglycemia in initial stages that gradually leads to insulin resistance[46]. The glucose intolerance was estimated on this PCOS model($p<0.05$), finding that rats showed hyperglycemic tendencies as it correlates with this other study[47] and in the current study it is perceived that the almond oil treated group, to have returned to normo-glycemic condition from their hyperglycemic condition. The effect produced by almond oil may be due to the high content of components such as the monounsaturated fat (MUFA's), vegetable protein, phytate, and phenolic content[48].

Due to excessive production of androgens in PCOS, it interferes with the process of follicular maturation and selection of dominant follicles during ova formation[49]. It also encourages early stages of follicular growth in ovary leading to the insulin resistance and fat distribution. As it is clear in this current study, there is a significant elevation in testosterone levels ($p < 0.05$) in letrozole treated group in contrast to control group. These are the consequences of the accumulation of androgens as the turning over of androgen substrates is blocked by letrozole and the elevated levels of testosterone in peripheral blood can be the reason for prolonged diestrus phase[50]. When treated with almond oil, testosterone levels were normalized which may be due to reduced levels of free androgens[51] as there is high concentrations of MUFA's and zinc present in almond oil. These components have been essential for the reproductive health.

One of the consequences of PCOS is dyslipidemia and the imbalances in lipid profile are attributed to hyperandrogenemia. Present study exhibited similar results in lipid profile as showed in other studies[52]. PCOS induced group showed significant increase in total cholesterol (TC) ($p < 0.001$), triglycerides level (TG's) ($p < 0.05$), LDL ($p < 0.001$) and decrease in HDL ($p < 0.05$) levels too. Almond oil displayed its antihyperlipidemic action by considerably decreasing serum TC, TG's, LDL while slightly decreasing the HDL levels.

The sections of ovary from untreated control group showed healthy follicles with oocyte at different stages of development. Ovarian morphological changes in letrozole treated group presented more number of follicular cysts, attenuation of granulosa cell layer, and hyperplasia of theca layer. It also exhibited numerous sub capsular cysts and corpora lutea were completely absent indicating anovulation. Few follicles were even observed at their early stages of development. Additionally, they were also accompanied with primary atretic follicles containing fluid filled antrum. These histological findings indicate the presence of biologically active levels of hormones such as FSH, increased LH, and lack of interaction between granulosa and theca cells[24]. Almond oil treatment showed protective effect to some extent where antral follicles were seen with secondary atretic follicles and few cystic follicles.

CONCLUSION

In conclusion, the results based on the biochemical assessment, histological findings and vaginal smears in this study demonstrates that prolonged use of almond oil can have beneficial effects in PCOS phenotypes by producing an antiandrogenic effect. The incision of PCOS has assisted in an impressive rise of scientific interest in this disorder, which should be further promoted and guided to improve individualized clinical approaches and, hence the therapeutic strategies. Almond oil is beneficial as it restores the lipid profile, glycemic status, testosterone as well as ovarian morphology in Letrozole treated rats. These effects may be connected to its multiple pharmacological activities like antiandrogenic, antihyperlipidemic, and hypoglycemic effects which could be useful in managing PCOS condition and hinder ovarian cell dysfunction, ovulation and hence improving fertility.

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