

## The effect of extract of *Ziziphora tenuior* L. On rats with polycystic ovary syndrome through tumor necrosis factor $\alpha$ (TNF- $\alpha$ )

### Authors:

Solmaz doostikhah<sup>1</sup>, latifeh karimzadeh bardeei<sup>1</sup>, Mohammad Nabiuni<sup>2,\*</sup>

<sup>1</sup>Department of Animal Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran.

<sup>2</sup>Department of Cellular and Molecular Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran.

### Corresponding Author:

Mohammad Nabiuni

Department of Cellular and Molecular Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran.

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### ABSTRACT:

**Background:** polycystic ovary syndrome to appear by lack of chronic ovulation, systematic inflammation and hyperandrogenism, is the most common endocrine dysfunction among women of reproductively age. *Ziziphora* plant due to having Pulegone, flavonoid and anthocyanin, acts as an anti-inflammatory and antioxidant. Tumor necrotic factor (TNF- $\alpha$ ) is effective in regulation the normal activity of ovary. The high rate of TNF- $\alpha$  causes the progress of polycystic ovary syndrome. In this research, we tried to consider the effect of *Ziziphora tenuior* L. Extract on TNF- $\alpha$  and also the symptoms of polycystic ovary syndrome. **Methods:** In this particular study, a total of 60 female adult Wistar rats were divided into three groups: control (n=12), PCOS (n=12), and *ziziphora*-treated PCOS (n=36). The induction of PCOS was achieved by injecting estradiol valerate at a dosage of 2 mg/kg. The PCOS rats were further divided into control and experimental groups, with the experimental group receiving daily intraperitoneal injections of *ziziphora*. After a period of 60 days following the induction of the syndrome, the ovaries were collected for histological and immunohistochemical evaluation. The levels of serum IL-6 and CRP were determined using an ELISA kit. The obtained data were then analyzed using In-stat through one-way analysis of variance (ANOVA), with a significance level of  $p < 0.05$ . **Results:** The dissections of the ovaries revealed a significant reduction in the thickness of the granulosa layers and an increase in the number of corpus luteum in the *ziziphora*-treated group compared to the PCOS group. Additionally, the levels of inflammatory markers such as IL-6 and CRP significantly decreased in the groups treated with *ziziphora* compared to the PCOS groups. In terms of the immunohistochemical analysis, the expression of TNF- $\alpha$  in the granulosa layer, follicular fluid of follicles, and ovarian cysts in the PCOS group was found to be higher than that in the control group. However, the expression of this factor in the ovaries treated with *ziziphora* was decreased. **Conclusion:** This research study demonstrates that the anti-inflammatory and antioxidant effects of *ziziphora* on PCOS may be attributed to its inhibitory effect on the expression and levels of TNF- $\alpha$ , IL-6, and CRP.

**Keywords:** follicular fluid, ovarian cyst, granulosa layer, TNF- $\alpha$ , IL-6, immunohistochemical.

### INTRODUCTION:

Polycystic ovary syndrome or PCOS is a very common hormone disorder among women of reproductivity age (15 to 49 years old), and its prevalence rate is around 5-10% of women(1). The most important feature of this syndrome is disorder in the normal ovulation process followed by disorder in the level of several other hormones, increasing the rate of C-reactive protein (CRP), LH hormones, estradiol and testosterone, while decreasing the rate of FSH and Progesterone hormones. This heterogeneous disease is detected through a spectrum with infertility demonstrations such as unnormal morphology of polycystic ovary in pelvic sonography, menstruation cycle disorder and infertility; and at the other end of the spectrum emerges with metabolic and inflammatory symptoms like overweight and hyperandrogenism. PCOS

pathophysiology with insulin resistance – means dysfunction in insulin performance in Glucose take-in and metabolism -has been started which is followed by Glucose intolerance(2). Insulin performance disorder and the increase in its consequent level (3) cause decreasing the synthesis of the two important binding proteins means insulin-like growth factor binding protein (IGFBP) and sex hormone globulin binding (SHBG); the first of them is connected to IGF-1,2 and the second to Androgens. Decreasing these proteins results in increasing the free ligands density and IGF-1,2 increasing causes androgen production through increasing Luteinizing hormone(LH) (4). Insulin in high densities acts Insulin-like growth factor 1 (IGF-1) receptors (5,6). Insulin tolerance cause hyperinsulinemia, dyslipidemia, blood pressure and increased risk of being affected by Diabetes type 2 and

coronary artery disease. Increased level of CRP as an index of low grade chronic inflammation can predict the danger of suffering to heart coronary artery disease and diabetes type 2 (7). It's observed that CRP in the people affected by Diabetes type 2 is increased (8) and low grade chronic inflammation markers can predict the individuals being at the risk of affection of diabetes type 2 independently (8,9).

PCOS may be regarded as a metabolic disorder having direct relation with Low grade inflammatory. Classic leucine-rich glioma inactivated 1 (LGI) markers – like CRP, Interleukin 1 (IL1)-, Macrophage Migration Inhibitory Factor(MIF), Monocyte Chemoattractant Protein-1 (MCP1), Tumor Necrosis Factors path  $\alpha$  (TNF- $\alpha$ ) and Matrix Metalloproteinase 2 (MMP2) are increased in PCOS.

Polycystic ovary syndrome is a kind of syndrome with complex endocrinic, metabolic and genetic disorders which is determined by chronic lack of ovulation, polycystic ovary and biochemical-clinical demonstrations such as hyperandrogenism. This syndrome has considerable negative effects on body physiology and metabolism such as insulin tolerance, hyperinsulinemia, gastric obesity, and high blood pressure, and in long time causes Diabetes type 2, endometrial hyperplasia and cardiovascular diseases.

TNF- $\alpha$  is a necrotic factor that playing a considerable role in regulation normal performance of ovary in the follicular growth. This factor is expressed in oocyte, normal and atretic granulosa cells, and locally by lutein granulosa cells playing role in forming the theca layer; because it causes passing from G0 to G1 in cellular cycle of inner theca layer cells and reproducing them(6,10). An expression more than TNF- $\alpha$  in rat causes progression of polycystic ovary syndrome alongside with induction of apoptosis in Antral Follicle Granulosa cells and stimulating the mitotic activity of interstitial theca layer cells and increasing the number of steroidogenic cells as well(2,3). So in those women with polycystic ovary syndrome, the diameter of granulosa layer is decreased while the diameter of theca layer is increased(10). *Ziziphora tenuior* L. plant, scientifically named *Ziziphora tenuior* L. belonging to the Lamiaceae family, is of the local plants of Iran used for treating the stomach diseases in traditional way. The most important compound of this plant is Pulegon. Anti-inflammatory and anti-pain effects are well known; so that can be used in treating fever, menstruation pains and stomach tonus(11–13). Several different researches showed a reduction in the growth of malignant tumors up to 32.6% and of cancerous tumors up to 47.55% by treating with this plant which is attributed to Pulegone. The anti-inflammatory effects of this plant are because of restraining Toxicity Acetic Acid and lipid peroxidation, chelate metals involved in oxidation and revival, the anti-inflammatory effects of this plant weaken the processes resulting in producing active Oxygenate compounds and hence, decrease the inner-cell oxidative stress in the rats that were inflammation-induced. Along with decreasing the oxidative stress rate resulting from decreased secreting of Neutrophils, the

negative regulation of IL-1B and decreased production of Prostaglandins are effective in restraining the inflammation.

Depending on the remedial effects of traditional plants in treating many inflammatory diseases, and regarding the reports emphasizing the role of Lamiaceae in negative regulation of the signaling paths resulting in inflammation, we tried to study the effects of mountain *Ziziphora tenuior* L. on the levels of hormones on the Hypothalamus-Pituitary axis and also, the changes of ovary tissue in rats affected by PCOS. Regarding the fact that Estradiol Valerate causes the induction of polycystic ovary syndrome in rats, along with causing inflammation on one side, and the fact that the mountain *Ziziphora tenuior* L. has anti-inflammatory effects on the other side, we studied the anti-inflammatory effects of this plant through the TNF- $\alpha$ .

## **MATERIALS AND METHODS:**

**Animals:** a total of 60 female Wistar rats, with an approximate weight of  $170 \pm 20$  gr, were utilized for the present investigation. The animals were maintained under standard laboratory conditions, adhering to a 12-hour light/dark cycle and a temperature range of 26–28°C, which were upheld until the conclusion of the study. The animals were placed in these conditions one week prior to the initiation of the research. The animal enclosures were kept clean, and sustenance and water were provided on a regular basis each day. All experiments conducted in this study were performed in accordance with the guidelines for animal research set forth by the national institutes of health and were duly approved by the Kharazmi university committee on animal research (KHU 616-9165).

**Study design and treatment:** the rats were distributed into three distinct groups, namely the control group (n=12), the group with polycystic ovary syndrome (PCOS) (n=12), and the experimental group treated with *Ziziphora tenuior* L. (n=12). In order to induce PCOS, the experimental group was administered 2 mg/kg BW estradiol valerate through intramuscular injection, following a single step process (14).

After a span of 60 days, three rats were anesthetized using chloroform to ensure the successful injection of PCOS. The induction in rats was then compared with the control group, which did not receive any injections. To ascertain the appropriate dosage of *Ziziphora tenuior* L., the concentration of a substance that caused the demise of 50% of mice, known as LD50, was taken into account. In this particular study, the concentration of LD50 was determined to be 1000 mg/kg BW *Ziziphora*. It is essential to note that *Ziziphora tenuior* L. is hydrophilic and soluble in water. Concentrations of 100, 150, and 200 mg/kg of body weight were selected as the treatment. After a treatment duration of ten consecutive days utilizing *Ziziphora tenuior* L., the rats were terminated via chloroform inhalation, subsequently leading to the isolation of the ovarian samples. Following the isolation of the ovaries for histologic and histochemistry techniques, they were then subjected to Bouin's fixative and paraformaldehyde. serological

analysis was conducted in order to quantify the levels of serum IL-6 and assess hormonal changes. Three groups were immersed in paraformaldehyde for a duration of 12 hours to enable qualitative evaluation of immunohistochemistry. Subsequently, the ovarian samples were molded with paraffin and sliced into sections with a diameter of 4  $\mu$ m. These sections were then placed on glass slides coated with 0.1% polylysine. Following a 24-hour incubation in the incubator, the paraffin was eliminated using xylene and a descending series of alcohol grades, respectively. To facilitate antigen retrieval, the tissue sections were subjected to 10mm citrate buffer with a pH of 6 at a temperature of 90°C for a period of 20 minutes. In order to eliminate non-specific binding sites of the primary antibody, the slides were rinsed with PBS buffer water. Subsequently, the slices were incubated in wet chambers with diluted TNF monoclonal (50:1)4% PBS-BSA (phosphate-buffered saline-bovine serum albumin) for a duration of 24 hours at 4°C. Following a wash in peripheral blood smear (PBS), the slides were immersed in a solution of 0.3% hydrogen peroxidase in methanol for 10 minutes to inhibit cell peroxidase activation. The slides were then washed again and incubated with a secondary antibody for TNF at room temperature in a humidified chamber for one hour. Detection was accomplished using the diaminobenzidine (DAB) dye and an immunohistochemistry accessory kit, resulting in the formation of a brown precipitate.. Hematoxylin was

employed as the counterstain for the staining procedure. The analysis of the data was conducted using a light microscope. The H-score system was established based on the proportion and intensity of brown-stained cells. For the assessment of the H-score, ten fields were randomly selected at a magnification of 400, and the staining intensity of each slide was assigned a score of 0, 1, 2, or 3, corresponding to the presence of negative, weak, intermediate, or strong brown staining, respectively(15).

Peripheral blood serum was isolated by centrifuge for 5 minutes speeds of 2000 rpm; and CRP and LH, Follicle stimulating hormone (FSH), testosterone, progesterone and estradiol was measured in Chemiluminescent immunoassay (CLIA) method.

For statistical analysis, image j, one-way ANOVA with post-hoc Tukey test were used p-value less than 0.05 was considered significant.

### RESULTS:

Control and estradiol valerat-induced animals were weighted, and it was shown that in addition to a significant increase in body weight ( $p<0.001$ ) in the induction group compared to the control group, the increase in abdominal fat was observed macroscopically. After intraperitoneal injection of *Ziziphora tenuior L.* for 10 days, abdominal fat tissue was modulated and reduced in body weight ( $p<0.001$ )(Table 1).

**Table1.Hormone concentration,ovarian and body weight of rats in the control,PCOS and treated with *Ziziphora tenuior L.* groups(n=12).In all groups,control was compared with PCOS group and *Ziziphora tenuior L.* treated PCOS was compared with PCOS group.**

Groups	FSH(ng/ml)	LH(ng/ml)	T(ng/ml)	P4(ng/ml)	E2(ng/ml)	Ovarian weight(mg)	Body weight(g)
Control	850±28.86	2.8±0.15	0.5±0.05	117±3	0.029±0.005	11.98±0.45	157.2±7.36
PCOS	**687.82±40.44	5.5±0.56**	***1.12±0.06	55±2.8**	***0.052±0.005	19.78±0.2**	215.5±12***
ZTE100mg/kg	686±3.48	2.86±0.1**	**0.74±0.03	62±2.3	*0.04±0.002	16.58±0.26**	205±15.67**
ZTE150mg/kg			***0.69±0.02	70±0.5*			
ZTE200mg/kg	836±18.55*	***2.46±0.09	***0.69±0.03	72±3.9*	*0.037±0.001	13.26±0.19	187.19±7.56**

**E2:17 $\beta$ -estradiol;T:Testosterone;LH:Luteinizing Hormone;P4:Progesterone;FSH:Follicle Stimulating Hormone;PCOS:Polycystic Ovary Syndrome.\*\*\* $p<0.001$ ,\*\* $p<0.01$ ,\* $p<0.05$**

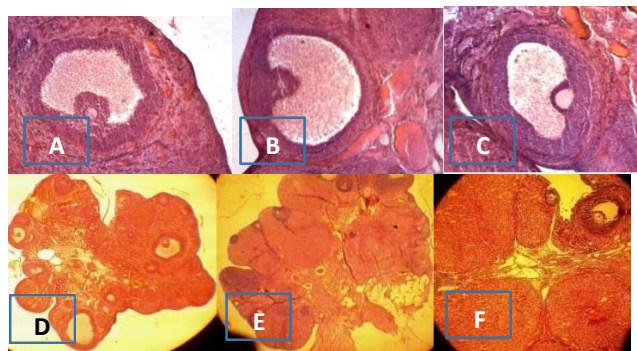
After euthanizing the rodents, extracting blood from the cardiac region, performing incisions in the abdominal area and peritoneum, an endeavor was undertaken to extract the ovaries, surplus adipose tissue, and oviduct tubes, followed by the weighing of the ovaries using refined scales (Sartorius-Germany). The average ovarian weight exhibited a noteworthy increment in the PCOS cohort when juxtaposed against the control group ( $p<0.01$ ). Amplifying the magnitude of follicular fluid and ovarian stroma might contribute to the accumulation of weight in the ovaries observed in PCOS subjects in comparison to the control group. Notwithstanding, the mean ovarian weight in the treated samples exhibited a reduction in contrast to the PCOS group

( $p<0.001$ )(Table).

Chemiluminescent immunoassay (CLIA) showed a significant increase ( $p<0.05$ ) in the levels of testosterone, estradiol, and LH in the PCOS group and a significant decrease ( $p<0.05$ ) in the levels of progesterone and FSH versus the control group. In this research, the administration of different doses of *Ziziphora tenuior L.* in PCOS rats for 10 consecutive days to achieve positive results. different doses of *Ziziphora tenuior L.* caused a significant decrease in the levels of estradiol, testosterone, and LH and a significant increase in the levels of progesterone and FSH, Due to the appearance of corpus luteum in the *Ziziphora tenuior L.*- treated ovaries (Table1).

To determine changes in follicular development, follicles were divided into six groups including primordial follicles (PMF), primary follicles (PF), preantral follicles (PAF), antral follicles (AF), cystic follicles (CF), and corpus luteum (CL).

In the ovaries of individuals with polycystic ovarian syndrome (PCOS), a notable presence of numerous small follicles and large cystic follicles, accompanied by a thin granulosa layer, was observed, which is a characteristic feature of PCOS. Conversely, the presence of corpus luteum was not observed in the PCOS group. In contrast, the control group exhibited an absence of ovarian cysts and an abundance of corpus luteum, indicating normal ovulation within this cohort. Consequently, the documented observation substantiated the development of cysts and the cessation of follicular growth following a 60-day treatment regimen involving estradiol valerate. This effect was attributed to the absence of normal ovulation, resulting in cyst formation and the disruption of normal follicular growth distribution (Figure 1).



**Figure1. Histological analysis of normal ovaries which had some corpus luteum (A,D) compared with PCOS including cystic follicles (B,E) and ovaries treated with *Ziziphora tenuior L.* showed decreased theca layers Graafian follicle (C,F). the rats ovarian tissues were stained with hematoxylin and eosin., and some ovulation. AF:atretic follicle, CF:cystic follicle, CL:corpus luteum, GL:granular layer, TL: theca layer, GF: Graafian follicle.**

In morphological studies in rats treated with *Ziziphora tenuior L.*, an increase in the number of primordial and preantral follicles and corpus luteum was observed, and a significant decrease in the number and size of cysts compared to the PCOS group. injection of high doses of *Ziziphora tenuior L.* (200mg/kg BW) showed almost no cysts were found. also, the present number of corpus luteum in this group shows their ovulation. The size of the follicles in the ovaries affected by Polycystic Ovary Syndrome (PCOS) exhibited a noteworthy increase in comparison to the control group and the PCOS group ( $p<0.001$ ). Furthermore, there was a significant reduction in the size of the follicles when comparing the groups treated with *Ziziphora tenuior L.* (100mg/kg BW) and the PCOS group ( $p<0.001$ ). However, the administration of a higher dosage of *Ziziphora tenuior L.* (150,200mg/kg BW) did not result in a significant decrease in follicle size, as indicated by Table 2.

**Table 2. ovarian follicular diameter( m)(n=12). In all groups, control was compared with PCOS group and *Ziziphora tenuior L.* treated PCOS was compared with PCOS group. PMF: Pri-mordial follicles; PF:Primary Follicles; PAF:Pre-Antral Follicles; AF:Antral Follicles; CF:Cystic Follicles; CL:Corpus Luteum.\*\*\* $p<0.001$ ,\*\* $p<0.01$ ,\* $p<0.05$ .**

Groups	PMF	PF	PAF	AF	CF	CL	Follicle diameter
Control	44±0.1	20±0.1***	28±0.1***	17±0.1***	0±0***	10±0.1*	560±0.8***
PCOS	41±0.05	11±0.05	16±0.05	3±0.15	16±0.1	4±0.1	721±0.3
ZTE100mg/kg	47±0.2***	13±0.1	19±0.15	8±0.15	10±0.1***	17±0.1***	651±0.4***
ZTE150mg/kg	50±0.05***	14±0.11***	24±0.1***	9±0.11***	9±0.1***	18±0.1***	620±0.3***
ZTE200mg/kg	55±0.2***	18±0.5***	44±0.15***	10±0.1***	6±0.1***	20±0.2***	610±0.05***

**IL-6 assay:** in this study, PCOS induction led to a significant rise in IL-6 inflammatory index( $p<0.001$  vs. control rats). the effect of *Ziziphora tenuior L.* (100,150,200mg/kgbw)on the level of IL-6 in PCOS rats was examined for 10 days after complete induction of PCOS. our study indicated that *Ziziphora tenuior L.* reduces IL-6 content in the *Ziziphora*- treated groups as compared to the PCOS group. It also indicated that *ziziphora* with a dose of 200 mg/kg BW *Ziziphora tenuior L.* is more effective than the dose of 100 mg/kg BW(Table 3).

**Evaluation of changes in CRP:** In addition to inflammatory changes CRP levels were measured in rats with PCOS.CRP is a member of the pentraxins family. As the most sensitive inflammatory indices, CRP plays an important role in immune response.PCOS induction led to a significant rise in this systemic inflammatory index, and its reduction in rats treated with *Ziziphora tenuior L.* was significant. Injection of *Ziziphora tenuior L.* showed a significant decrease in the levels of CRP in the *ziziphora*-treated rats( $p<0.001$ ).

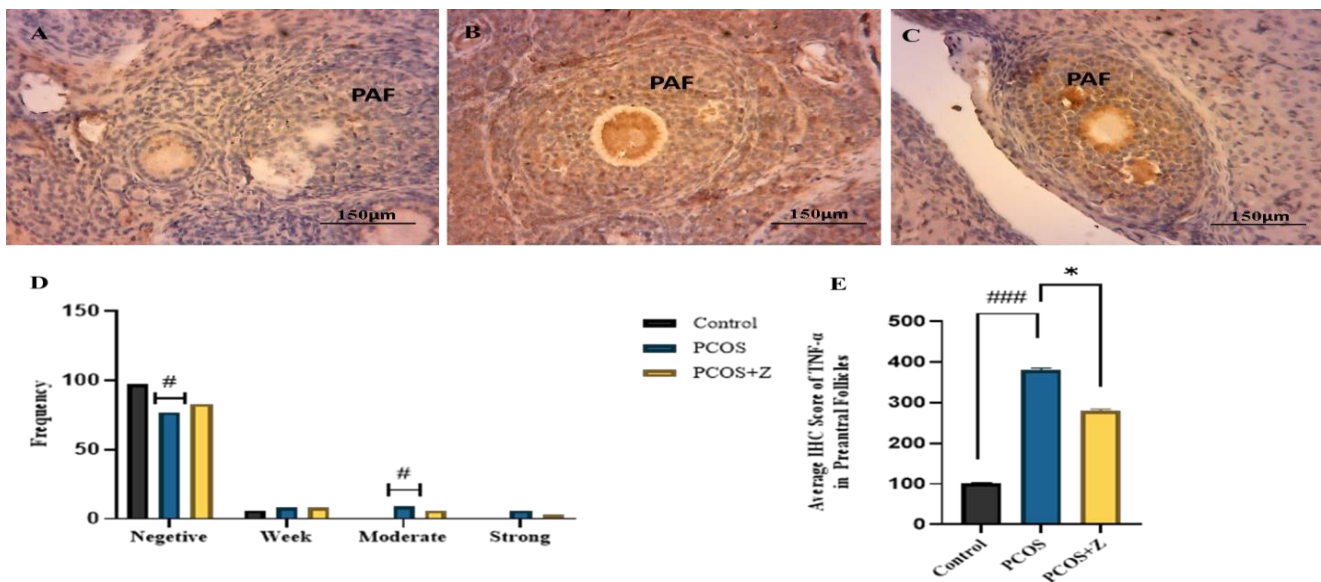
**Table 3. The follicles morphometric characterize and IL-6 and CRP compared to the control group, PCOS and treated with *Ziziphora tenuior L.* (n=12)**

Groups	Stroma	Preantral	Grannulosa	Theca	CRP(pg/ml)	IL-6(pg/ml)
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Control	115±2.6	12±2	11±3	10±2	0.145±0.002	0.587±0.008***
PCOS	126±2.5*	53±3.2***	116±3.5***	13±2.1***	0.27±0.010**	0.770±0.006
ZTE100mg/kg	112±2.5	58±3	88±3.5***	12±2*	0.23±0.017	0.768±0.007
ZTE150mg/kg	90±4.1***	45±2	56±5***	16±2.3***	0.20±0.028	0.677±0.002***
ZTE200mg/kg	103±2**	38±2.9	22±2.5***	10±2	0.19±0.005*	0.6±0.02***

Immunohistochemistry analysis: given the significance of TNF- $\alpha$  expression in the stimulation of proliferation and steroidogenesis in the theca cells of the ovarian follicle, this study investigated the levels of TNF- $\alpha$  expression in both pre-antral and antral follicles located in the granulosa layer of PCOS ovaries. However, in normal ovaries and those treated with *Ziziphora tenuior* L., the expression of this factor in the granulosa layer of antral and pre-antral follicles was comparably lower than that observed in the PCOS group (see figure 2). This enzyme is not expressed in the follicular theca but in follicular fluid, a brown precipitate is obvious. expression levels of TNF- $\alpha$  in several layers of ovarian follicular cells treated with *Ziziphora tenuior* L. showed reduction compared with the PCOS group. In contrast, granulosa layers in the pre-antral, antral follicles, and the cells surrounding the oocyte expressed less TNF- $\alpha$

(Figure 3). In the group of individuals with polycystic ovary syndrome (PCOS), a significant level of immunoreactivity to TNF- $\alpha$  was observed in the ovarian stroma, preantral follicles, and granulosa layer ( $p < 0.001$ , ( $p < 0.05$ ). Conversely, a weak expression of TNF- $\alpha$  was noted in the theca layer ( $p < 0.001$ ) when compared to the control group. The presence of TNF- $\alpha$  in stromal cells within the PCOS group treated with *Ziziphora tenuior* L. displayed a noteworthy reduction ( $p < 0.001$ ,  $p < 0.01$ ,  $p < 0.05$ ) in comparison to PCOS. Furthermore, the intensity of immunostaining in the theca layer of the *Ziziphora*-treated PCOS group was less pronounced than that of the PCOS group ( $p < 0.001$ ,  $p < 0.05$ ). In the *Ziziphora tenuior* L. treated PCOS group, TNF- $\alpha$  exhibited a diminished expression in both granulosa and preantral follicles in comparison to the PCOS group ( $p < 0.001$ ,  $p < 0.05$ ) (Table 3).



**Figure 2.** photomicrographs of immunohistochemical expression of TNF- $\alpha$  in the primary and secondary follicles. (A) normal ovary; (B) PCOS group; (C) *Ziziphora tenuior* L.- treated PCOS group. (D) semiquantitative expression of TNF- $\alpha$  in negative, weak, moderate and strong level in normal, PCOS and Z-treated groups. (E) semiquantitative analysis of TNF- $\alpha$  average expression in normal, PCOS and Z-treated groups. Results are mean  $\pm$  SEM (n = 7).

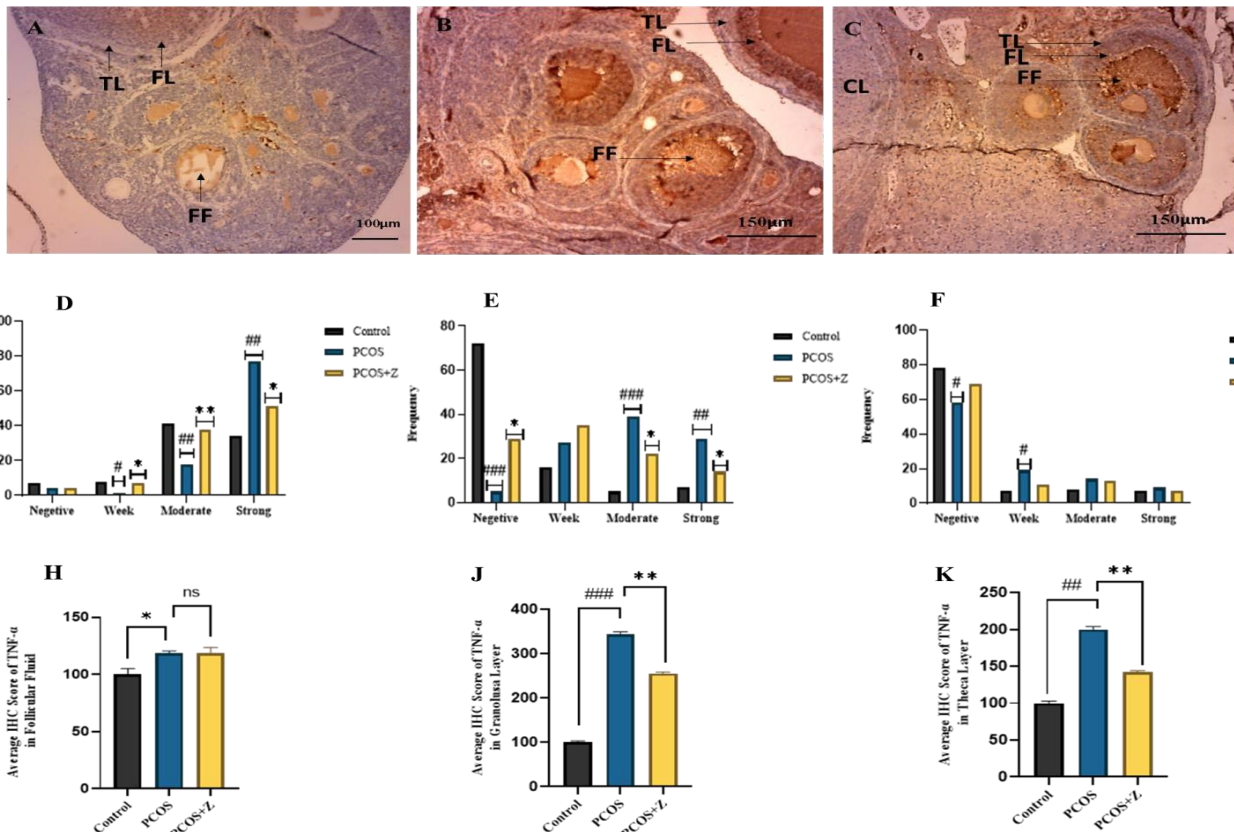


Figure 3. Photo micrographs of immunohistochemical expression of TNF- $\alpha$  in follicular and theca layers and graffian follicles fluid. A) due to high levels of angiogenesis, the density of follicular fluid, as well as TNF- $\alpha$  expression are high. the thick theca and granulosa layers indicate high expression levels of TNF- $\alpha$  in PCOS group. B, C, D) decreased TNF- $\alpha$  expression levels in follicular fluid and granulosa layer in *Ziziphora tenuior* L. groups. Results are mean  $\pm$  SEM (n = 7).

## DISCUSSION:

In this study, we examined the effect of *Ziziphora tenuior* L. plant (having flavonoid elements with anti-inflammatory properties) on improving polycystic ovary symptoms induced by Estradiol Valerate through tumor necrotic factor path.

Tjahyadi in 2022 explained that increasing the secondary follicles number results in stimulating the increase of tonic LH without the rise step. Consequently, it results in creating antral follicles without ovulation and hence, creating cysts in women(16). Barath reported after inducing PCOS in rats by Estradiol Valerate, some cysts were created in ovary that the source of them was of atresic antral follicles and have features like degenerated granulosa cells layer and thick layer compared to control group(17). In this study, besides, we induced this animal model using Estradiol Valerate which is more similar to human models comparing to other induction ways regarding creating ovary's tissue changes, hormone level and metabolic changes. Ovary's tissue changes in PCOS group in this study - corresponding to Tjahyadi and Barat s' results - showed a successful expression of this syndrome and creating defect in development process of follicular groups in mature rats comparing to control group. The findings of this investigation are consistent with the research conducted by Xing et al. in the year 2022. Their study demonstrated a noteworthy elevation in the levels of proinflammatory cytokines (specifically IL-6 and IL-18) in females diagnosed with polycystic ovary

syndrome (PCOS). Moreover, these cytokine levels were observed to be positively associated with indicators of insulin sensitivity. The administration of metformin exhibited a beneficial impact on both the metabolic and endocrine profile, as well as menstrual function. Additionally, the use of metformin was also linked to the occurrence of spontaneous pregnancies.(18).

According to Aminzare et al. Studies in 2022, it seemed that increasing blood veins due to Angiogenesis (increasing the expression of vascular endothelial growth factor and isoenzyme 2 cyclooxygenase) in Stroma and theca layer cause an increase in Androgen production, thickening this layer and steroidogenesis progress(19); increased thickness of theca layer is dependent both on hypertrophy of cells of this layer, and an increasing the number of Interstitial Theca cells in this layer(20). As Baharfar says that insulin and semi-insulin factors are autocrin' and paracrine's regulators of these cells in humans and rats, and can multiply and stimulate the production and activity of steroidogenesis of these cells and, hence, result in increasing androgen synthesis in theca and granulosa cells. Granulosa cells receive testosterone in shape of oestrone from theca layer cells, and convert them into estradiol; by growing follicles, more estradiol will be made(21). So Querol explained that women affected by polycystic ovary syndrome have more estradiol concentration than normal ones(22). In this study, beside increasing the level of testosterone and serum estradiol in PCOS rats, we observed a noticeable decrease in the

serum level of progesterone in this group due to the decreased produce of corpus luteum and increasing the number of the follicles haven't ovulated and have created cysts; which was corresponding with Qurol' and Yang's studies regarding the changes of serum level of progesterone in PCOS samples(23,24). Considering the restraining effects of flavonoids – Thymol and Carvacrol (of important compounds in the extract of this plant family) – on cyclooxygenased enzymes could say that *Ziziphora tenuior L.*, probably, plays role in improving the ovarian tissue symptoms in PCOS rats treated by hydroalcoholic extract of this plant through restraining the production of theca layer cells(25).

Changing the hormone profile in this study as respectively as mentioned before, provide a field of considering *Ziziphora tenuior L.* extract's effects beside approving the syndrome induction in rats of Vistar race. Treating rats with different-concentration *Ziziphora tenuior L.* extract intraperitoneally resulted in modifying the level of mentioned hormones (decreasing LH, testosterone and Estradiol level; also increasing FSH and progesterone). The effect of these hormone changes on ovarian tissue emerged along with histological symptoms including emergence of corpus luteum and decreasing the amount of follicles in primary steps of follicular development.

The increased androgen cause a rapid growth in primary steps of follicular development and tonic LH (without increasing first phase at the time of ovulation) by applying stimulating effects on releasing gonadotropins (specially LH). Increasing tonic LH took into consideration from 2 aspects: Pandiyan pointed out the dependence of LH hormone on the fat rate of the body in his study; he attributed a decrease in this hormone production to a decrease in fat mass and body weight(26). In this study, we can attribute the body weight increase in PCOS group rats – depending on Loucks studies – to serum LH level increase. Because in the rats treated by *Ziziphora tenuior L.* extract, when decreasing the serum level of this hormone, the body weight of animals decreased considerably compared to PCOS group. From a different aspect we can say, according to Rabbah studies in 2022, there's a decrease in the required enzymes for cracking the follicular walls and ovulation(10). As observed in the *Ziziphora* -extract- treated samples an increase of corpus luteum number preceded by a decrease in this hormone level. The results of this study along with the studies accomplished by Perinelli, were a significant decrease of average LH in metformin-treating and also, improving the tissue symptoms of this syndrome in the ovaries of women affected by PCOS(6).

In accordance to Adamczak's studies in 2010, we can say that increasing body weight and serum level of CRP in polycystic group would be attributed to the increase of systemic inflammation rate in animal model rat(2). Fat tissue is the main source of producing inflammation-promoter cytokine including interleukine 6 which sets the secreta of CRP in liver(11,12). These results were shown in human by Luan. Luan observed in

2022 that the women affected by polycystic ovary syndrome have a higher rate of CRP compared to normal ones(11). On the other hand, dependent on Perinelli's study in 2022, the decrease of CRP accompanied by decrease of metabolic symptoms in PCOS samples treated by metformin(6). Also, Aminzarein 2022 explained that a decrease in systemic cytokine amount by honey bee's venom results in travelling the PCOS ovarian symptoms to the normal ovary(19). Depending on these studies and creating a link between them, can conclude that every drug like Metformin and honey bee's venom decreasing the amount of inflammation- promoter cytokines including CRP and/or IL-6, can play a role in decreasing PCOS symptoms. Therefore we may say that the improvement of PCOS symptoms such as polycystic ovarian tissue changes affected by CRP level decrease by *Ziziphora tenuior L.* extract The expression of cytokines (TNF- $\alpha$ , IL-6) derived from adipose tissue could potentially play a significant role in the initiation and perpetuation of low-grade chronic inflammation. To put it differently, the buildup of visceral adipose tissue might serve as a fundamental element that supports the manifestation of metabolic syndrome and low-grade chronic inflammation.(27).

Coppa et al.'s study showed that *Ziziphora tenuior L.* contents can restrain colitis in mice. Biochemical measuring showed that prescribing *Ziziphora tenuior L.* extract decreases the activity of MPO and also the TRABS concentration which are the main indicators of stress oxidative and colitis markers. The main point of his studies was the similarity of *Ziziphora* effect (mainly in 300 mg/kg BW concentration) with Prednisolone which well protects the animal against colitis well. Prednisolone restrains phospholipase A2 enzyme and thus, decreases the amount of Prostaglandins and Leukotrienes. So probably, *Ziziphora tenuior L.* has restraining effects on synthesis of releasing these inflammatory mediators including Cyclooxygenase 2(COX2), inos, NO, Nuclear factor kappa B (NF-KB), TNF- $\alpha$ , IL-10, IL-6 AND IL-1(3,4). As shown in Ciona's studies, the inflammation of lens protein would be significantly decreased by localized and gradual using Pulegone compound of *Ziziphora tenuior L.* plant(5,6). According to Whitaker et al. Studies, distinguished that the *Ziziphora tenuior L.* extract having phenol contents and flavonoids, has an anti-inflammatory activity through controlling inflammatory cytokines or inflammatory mediators including NF- KB, TNF-  $\alpha$ , IL-10, IL-6, IL-1, NO, inos and COX-2. So the extract of this plant has pharmacologic and remedial effects(23). Thymus serpyllum has different compounds that the main of them are Thymol and Carvacrol(19). These contents restrain Cyclooxygenase and stress oxidative enzymes and hence, resulted in decrease stress oxidative(1,28–30). Since *Ziziphora tenuior L.* and Thymus Serpyllum are from the same family and having similar contents, it seemed that both of them decrease stress oxidative in the same way. Flavonoids are other contents in

hydroalcoholic extract of *Thymus serpyllum*(19). It was shown in a study that Flavonoids restrain the growth and metastasis of Pulmonary Carcinoma in mice(3). In this study, the increase of CRP amount in PCOS samples shows the metabolic assumption induction of this syndrome showing that Estradiol Valerate increases the expression of inflammatory metabolite through affecting the Angiogenesis path and increasing VEGF expression. So *Ziziphora tenuior L* extract can paly a role in differentiation and maturing follicular cells and regard as a new drug for ovulation induction.

In order for considering the inflammation amount in studied groups, we used Necrosis tumor factor. After providing poly-L-Elysine layer, we used TNF- $\alpha$  antibody and observed changes. TNF- $\alpha$  expression change due to the *Ziziphora tenuior L* extract effect was considered by immunohistochemical technique. Presumably the cause of this factor expression was showed in polycystic ovaries is due to the inflammation. A decrease in TNF- $\alpha$  expression was showed in polycystic ovaries treated by *Ziziphora tenuior L* extract is due to the changes applied by this extract in TNF- $\alpha$  signaling path.

TNF-  $\alpha$  is expressed in inflammatary samples in a way that ROS causes the induction of stress oxidative and by activating NF-KB path results in increasing TNF-  $\alpha$  transcription and Insulin resistance. NF-KB is activated by many inflammation stimulus. In the samples treated by *Ziziphora tenuior L* extract having inflammation, TNF-  $\alpha$  created brown color. In the samples having more inflammation, the brown color intensified to the same extent.

**CONCLUSION:** the results of the present study is consistent with the previous studies indicating that *Ziziphora tenuior L* reduction effects of PCOS on ovarian, such as follicular sheath diameter increase,the increase in ovarian weight and increased TNF expression in ovarian tissue.

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**Authorship:**

M.N. and L.K. designed the study, S.D did the laboratory data, analyzed and interpret of data, draft the article, L.K. revised it critically for important intellectual content and approved the version to be submitted.

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Ethics approval and consent to participate

There are no “human subjects” in this study. All ethical considerations were taken into account following the Helsinki Convention and the observance of animal rights, and experiments were performed after the approval of the Ethics Committee of the University of Kharazmi (Ethical Code KHU

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