



## RESEARCH ARTICLE

# Therapeutic efficacy of *Trifolium pratense* L. on letrozole induced polycystic ovary syndrome in rats

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### ABSTRACT

Polycystic ovary syndrome (PCOS) is considered as one of the leading endocrine disorders during reproductive age in women. This study designed to determine the therapeutic effects of red clover (*Trifolium pratense*) on letrozole-induced PCOS *in vivo*. Forty female Sprague–Dawley rats were equally divided into five groups. Control group with a regular sexual cycle received normal saline (letrozole vehicle). Letrozole (1 mg/kg) was used to induce the PCOS to the rats in the treatment groups. After induction of PCOS, four treatment groups received the normal saline, or clomiphene citrate (1 mg/kg), or red clover extracts (500 or 750 mg/kg) for 30-days. After treatment, ovary and uterus were removed, weighed, and the ovaries were subjected to histopathological studies. Serum testosterone and estradiol levels, antioxidant activities, and lipid profiles were evaluated. Red clover extracts and clomiphene citrate decreased testosterone levels and showed a significant increase in estradiol levels in comparison to PCOS induced group ( $p < 0.05$ ). Red clover administration restored the GSH, SOD and CAT levels ( $p < 0.05$ ) and decreased the NO and MDA levels ( $p < 0.05$ ). Treatments caused no significant change in levels of TG, TC, and FBG factors when compared to PCOS induced group ( $P > 0.05$ ). However, red clover (750 mg/kg) significantly increased HDL and decreased LDL levels when compared to PCOS induced group ( $P < 0.05$ ). Treatment with red clover reduced ovarian weight, volumes of ovarian, medulla, cortex and number of cysts and increased number of oocytes compared to PCOS group. Both red clover and clomiphene citrate could treat the letrozole induced PCOS in rats; however, red clover indicated antioxidant activities more than clomiphene citrate. Red clover may be used for discovering anti-PCOS drugs with lower side effects.

## Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in females. PCOS affects an estimated 4 to 15% of women in their reproductive age (1, 2); however, the aetiology and pathogenesis of this syndrome remain poorly understood. The features of this syndrome are infertility, alopecia, irregular menstrual cycles, acne and hirsutism (3, 4), and also metabolic disorders (insulin resistance, obesity and dyslipidemia), type 2 diabetes, cancer, coronary heart diseases, and hypertension (5, 6).

Changing the habits of life, surgery, and medication are widely followed therapies for PCOS. The most recognized medications are clomiphene

citrate therapy, metformin, and tamoxifen (7, 8). There are a variety of side effects such as arthritis, muscle pain and psychological disturbance in drugs that have been used for management of PCOS (9, 10). Due to side effects caused by chemical drugs, the medicinal plants are gaining interest in the prevention and treatment of diseases (11–13). Therefore, the present focus is being laid on natural medicinal plants with minimum side effects.

Red clover with scientific name *Trifolium pratense* L. is one of 250 different species of the *Trifolium* genus, which belongs to the Leguminosae or Fabaceae plant family (14). Red clover has a wide variety of biological potential in traditional medicine such as anti-cancer, antioxidant, estrogenic and

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progestogenic activities (15, 16). The aerial part of this plant has four isoflavones including biochanin A, formononetin, genistein, and daidzein (17). These phytochemicals have shown a lot of interest due to their human health benefits. Red clover isoflavones are estrogen receptor beta, strong agonists. They have selective estrogen receptor modulator potential and consequence, display a positive effect on menopausal symptoms (18). In addition, isoflavones belong to phytoestrogen class are useful for the treatment of cardiovascular disease, osteoporosis, menopausal symptoms, and even cancer (19). Regarding to the effects of the red clover and its compounds on human health, the aim of this study was to evaluate the therapeutic efficacy of red clover on letrozole-induced polycystic ovary syndrome.

## Materials and Methods

### Herbal extract preparation

Red clover (*Trifolium pratense* L.) was collected from Yasuj (Iran) in June 2016. The plant identified in the Herbal Medicinal Research Center of Yasuj University of Medical Sciences (Voucher no. HMRC-r 15). Plant materials were extracted with 70% (v/v) ethanol by maceration method at room temperature for 24h.

### Grouping and treatments

In this study, adult female Sprague-Dawley rats were acquired from Shiraz Animal House and were kept in the room at controlled conditions of temperature  $25 \pm 2^\circ\text{C}$ ,  $56 \pm 5$  humidity and 12-h daily cycle for 7 days to be acclimatized. They were fed with the conventional pellet food (Behparvar Com., Iran) and water *ad libitum* during of study. All procedures of the study were performed according to the approved local Animals Ethics Committee (Protocol approval number: 150/2014).

Forty female Sprague-Dawley rats with regular estrus cyclicity were equally divided into 5 groups of 8 rats each. During the experiment, the rat's estrus cycle was determined on each group by vaginal smear using Giemsa stain to confirm the induction of PCOS. If the rats exhibited acyclic/irregular ovarian cyclicity, they were considered as PCOS positive. Induction of polycystic ovary syndrome was performed using letrozole (Femara, Novartis, Istanbul, Turkey) administered to the rats (1mg / kg dissolved in 0.9% NaCl solution) p.o. once daily for 21 consecutive days (20).

Animal models of PCOS has offered a useful method for pathophysiology study of the syndrome. Presently, these models including administration of Estradiol Valerate, DHEA, prepubertal androgen excess and letrozole have been developed. These models mimic mainly features of PCOS in women (21).

Letrozole is a potent non-steroidal aromatase inhibitor. It used as a PCOS model in some animal experiment with many histologic and biochemical finding similarity with human PCOS. In the most of the experimental research for inducing of PCOS using 400  $\mu\text{g}/\text{day}$  letrozole for 7-35 days (22).

Control or normal group with a regular sexual cycle that received normal saline (letrozole vehicle) orally for 21 days. All the experimental animals except control group were received letrozole. After 21 days, rats were divided into 4 groups as follows:

PCOS: rats were given 21 days letrozole and afterward 30-day normal saline. Standard: Animals received letrozole for 21 days and afterward administered with clomiphene citrate at a dose of 1 mg/kg in 0.9% NaCl solution per oral. Red clover (RC)-500: was given 21 days letrozole and afterward treated with 500 mg/kg red clover extract orally for 30 days. RC-750: was given 21 days letrozole and afterward treated with 750 mg/kg red clover extract orally for 30 days.

### Blood sampling and biochemical assessments

The animals were evaluated according to a relative number of leukocytes, epithelial and cornified cells, 24 hours after the last sex cycle. The animals were anesthetized with diethyl ether the last dose of the treatment after 24 h fasting and 3-5 ml of blood was collected by cardiac puncture. Finally, animals sacrificed and the ovary and uterus were removed, weighed and the ovaries were fixed 10% buffered formalin and processed for histopathological study.

Serum was separated immediately and kept in a freezer at  $-20^\circ\text{C}$  for an estimate of biochemical parameters. Serum testosterone and estradiol were measured with commercially available ELISA kits. The assays were performed according to the manufacturer's instructions.

The activity of superoxide dismutase (SOD) was determined by spectrophotometer. The SOD activity was expressed in unit's/mg protein (23). Catalase (CAT) activity was estimated according to the method Beers and Sizer by spectrophotometer. One unit of the enzyme was defined as m moles of  $\text{H}_2\text{O}_2$  degraded/min/mg of protein (24).

Glutathione (GSH) content was determined according to Jollow et al. (1974) method. The contents were expressed in micromoles/mg protein (25). MDA levels were estimated via thiobarbituric acid reacting substance (TBARS). The MDA levels were expressed in micromoles/mg protein (26).

Nitrate/nitrite was assayed according to the method of Green et al. (1982) method using Griess. The results were expressed as micromoles/mg protein (27).

Lipid profile [triglycerides (TG), total-cholesterol (TC), LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C)] and FBG were measured according to commercial Iranian diagnostics kits in autoanalyzer.

### Stereological study

#### *The volume of the ovary*

To estimate the volume of the ovary, the ovary was sectioned into isotropic uniform random (IUR) sections using the isotropic Cavalieri method and using a stereomicroscope connected to a computer, at the final magnification of 20X. A brief description is presented under Figure 8 (28). The volume was determined via the following formula:

Ovary volume =  $\Sigma ni = p \times T \times a(p)$ .

Where  $\Sigma ni = p$  considers as the total number of points superimposed on the images; T considers as the distance between the sampled sections, and  $a(p)$  considers as the area associated with each point.

### The volume of cortex, medulla, ovarian cysts

The point counting technique was used to compute the volume of the structures via the following rule:

Volume of the structures =  $\Sigma P$  (structure) /  $\Sigma P$  (total)

Where  $\Sigma P$  (structure) and  $\Sigma P$  (total) consider as the total points striking the aim structure and the total points striking the ovary sections, respectively.

### Estimation of the number of oocytes

The number of atretic oocytes, antral and preantral was determined using a light microscope. The ovary specimens from all experimental groups were sectioned into 5  $\mu$ m thicknesses, stained with hematoxylin-eosin (HE) and pathological-physiological structures were studied on a PC in conjunction with a (Nikon E200 microscope, Tokyo, Japan) with a 60  $\times$  oil immersion objective.

### Statistical methods

The collected data were statistically analyzed by one-way ANOVA followed by post hoc analysis. Tests were carried out in triplicate. The data were expressed as the Mean  $\pm$  S.D. The P value less than 0.05 considered as significant.

## Results and Discussion

In the present study was aimed to evaluate the therapeutic efficacy of red clover (*Trifolium pratense*) on letrozole-induced PCOS in rats, both red clover and clomiphene citrate could treat the letrozole induced PCOS in rats; however, red clover indicated

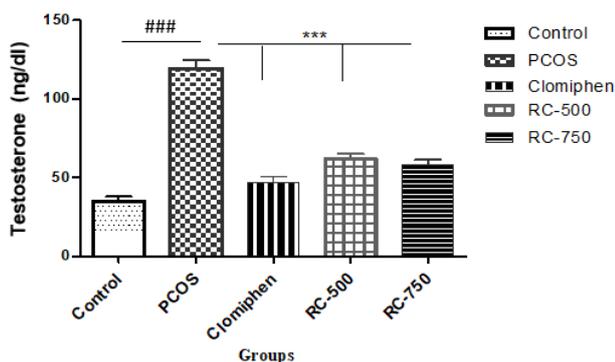


Fig. 1. Effect of red clover extract on serum concentrations of testosterone in letrozole-induced PCOS rats.

Control: Normal saline recipient; PCOS: Letrozole recipient; RC-500: Red clover 500 mg/kg recipient; RC-750: Red clover 750 mg/kg recipient. Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple. # denotes statistical significance in comparison to normal group rats \*denotes statistical significance in comparison to PCOS group rats ### p<0.001; \*\*\*P<0.001.

higher antioxidant activities than clomiphene citrate.

The serum level of testosterone was significantly increased in PCOS-induced rats group by letrozole

(p<0.001) at the terminal day of the study in comparison to control group. Red clover and clomiphene citrate (p<0.001) successfully restored

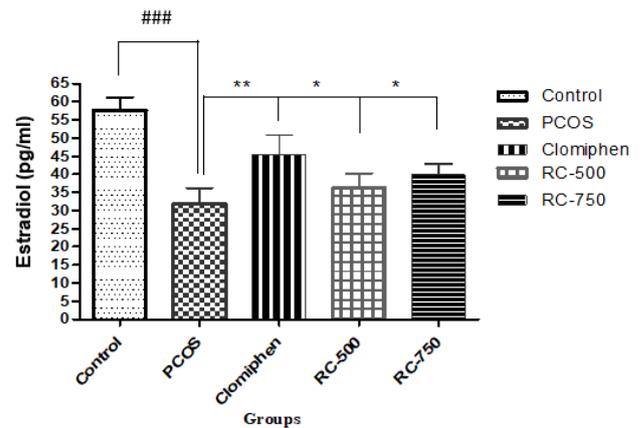


Fig. 2. Effect of red clover treatment on serum concentrations of estradiol in letrozole-induced PCOS rats.

Control: Normal saline recipient; PCOS: Letrozole recipient; RC-500: Red clover 500 mg/kg recipient; RC-750: Red clover 750 mg/kg recipient. Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple.

# denotes statistical significance in comparison to normal group rats \*denotes statistical significance in comparison to PCOS group rats ###p<0.001; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001

testosterone levels in comparison to PCOS induced group (Fig. 1).

Induction of PCOS indicated a significant

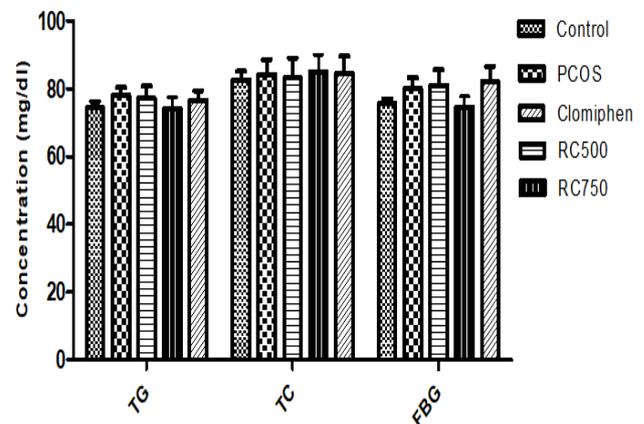


Fig. 3. Effect of red clover treatment on serum concentrations of TG,

TC and FBG in letrozole-induced PCOS rats. Control: Normal saline recipient; PCOS: Letrozole recipient; RC-500: Red clover 500 mg/kg recipient; RC-750: Red clover 750 mg/kg recipient. Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple.

decrease (p<0.001) in estradiol level at the last day of the study. Oral administration of red clover and clomiphene citrate showed a significant increase in estradiol levels in comparison to PCOS rats (Fig. 2).

In PCOS induced group, levels of TG, TC, and FBG increased; but it was not significantly (P>0.05) in comparison to control rats (Fig. 3). However, LDL and HDL levels were markedly restored (P<0.05) in comparison to PCOS induced group (Fig. 4).

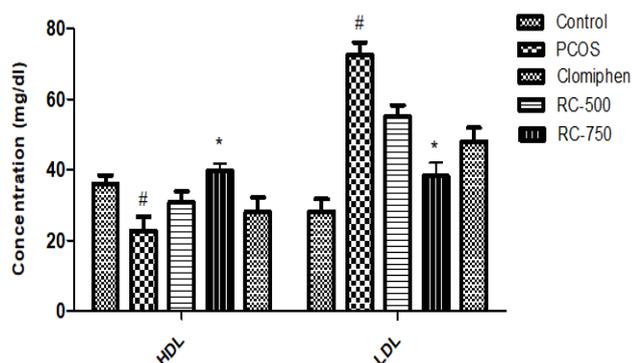


Fig. 4. Effect of red clover treatment on serum concentrations of HDL and LDL in letrozole-induced PCOS rats.

Control: Normal saline recipient; PCOS: Letrozole recipient; RC-500: Red clover 500 mg/kg recipient; RC-750: Red clover 750 mg/kg recipient. Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple.

# denotes statistical significance in comparison to normal group rats.

\*denotes statistical significance in comparison to PCOS group rats #p<0.05; \*P<0.05.

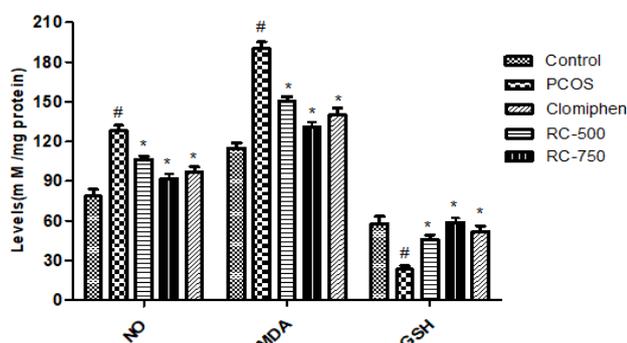


Fig. 5. Effect of red clover treatment on serum concentrations of NO, MDA and GSH in letrozole-induced PCOS rats.

Control; Normal saline recipient, PCOS; Letrozole recipient, RC500: Red clover 500 mg/kg; RC750: Red clover 750 mg/kg; Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple.

# denotes statistical significance in comparison to normal group rats.

\*denotes statistical significance in comparison to PCOS group rats.

In comparison to control rats, PCOS rats significantly indicated an elevation ( $p<0.05$ ) in NO and MDA levels. Treatment with red clover significantly ( $p<0.05$ ) decreased the MDA and NO levels in treatment rats (Fig. 5).

Administration of letrozole decreased ( $p<0.05$ ) the SOD, CAT activities, and GSH levels in PCOS rats in comparison to control rats. In comparison to vehicle-treated PCOS rats, red clover administration significantly increased ( $p<0.05$ ) the SOD and CAT activities ( $P<0.05$ ). Red clover successfully restores the GSH levels ( $P<0.05$ ) (Fig. 6).

In order to develop polycystic ovary syndrome, letrozole, a non-steroidal aromatase inhibitor was used. In this case, androgens cannot convert to estrogens and resulted in the elevation of androgen and consequently fall in estrogen levels (29). Besides, the increased level of testosterone in plasma, that resulted in prolonged diestrus phase and it was followed by an increase in body weight of PCOS group rats (30, 31). Administration of long-term

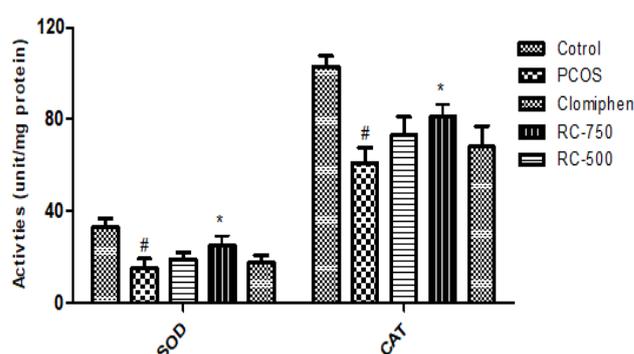


Fig. 6. Effect of red clover treatment on serum concentrations of SOD and CAT in letrozole-induced PCOS rats.

Control; Normal saline recipient, PCOS; Letrozole recipient, RC500: Red clover 500 mg/kg; RC750: Red clover 750 mg/kg; Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple.

# denotes statistical significance in comparison to normal group rats.

\*denotes statistical significance in comparison to PCOS group rats. #p<0.05; \*P<0.05.

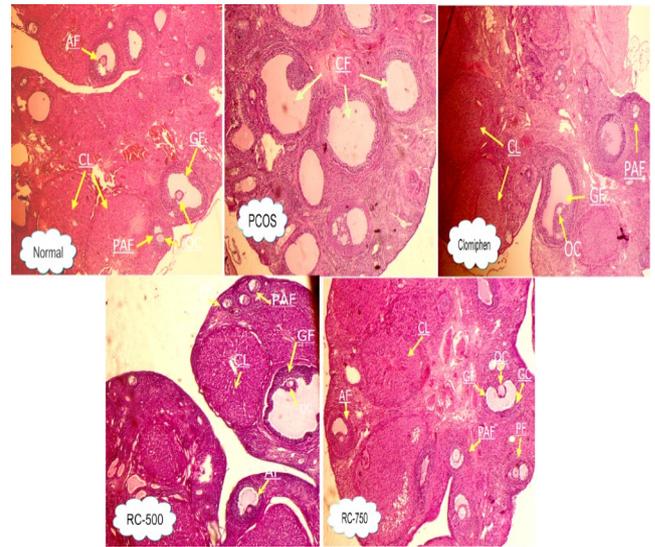
herbal extracts with phytoestrogens compounds can reduce plasma testosterone levels by the negative feedback effect on LH hormone (32). Administration of red clover extract was able to normalize testosterone and estradiol concentration in comparison to PCOS rats group. According to earlier reports, red clover with antiandrogenic properties and high content of phytoestrogens such as isoflavones in the plant may reduce androgen levels. Red clover isoflavones are weak agonists of estrogen alpha receptors (ER $\alpha$ ) and potent agonists of estrogen beta receptors (33). Moreover, the phytochemical compounds of red clover such as genistein and daidzein could interfere with steroid formation in rat adrenal and consequence decreased testosterone levels (34, 35). In addition, coumestrol, biochanin, phytoestrogens and zearalenol have inhibitory effects on steroidogenic enzyme activity of 17 $\beta$ -HSD which result in reduced androgens synthesis (36).

In the present study, treatments caused no significant change in levels of TG, TC, and FBG factors as compared to PCOS induced group. However, red clover (750 mg/kg) significantly increased HDL and decreased LDL levels when compared to in PCOS induced group. Letrozole-induced PCOS rats did not indicate significant changes in FBG, TC, and TG levels. The reason for this type of result was that letrozole did not have any effects on insulin signaling pathways (5). In addition, the letrozole-induced rodent model of PCOS did not show any metabolic aberrations such as adiposity, insulin sensitivity, and dyslipidemia, which were reported in parallel with some studies (21).

Oxidative stress is considered as an important pathological feature of PCOS. It causes a change in oxidant-antioxidant profile. Hence, women with PCOS indicate a decreased level of total antioxidant status (37). In the current study, oxidative stress markers (MDA and NO) were increased in PCOS animals. However, endogenous antioxidants (SOD, CAT and GSH levels) in blood were decreased.

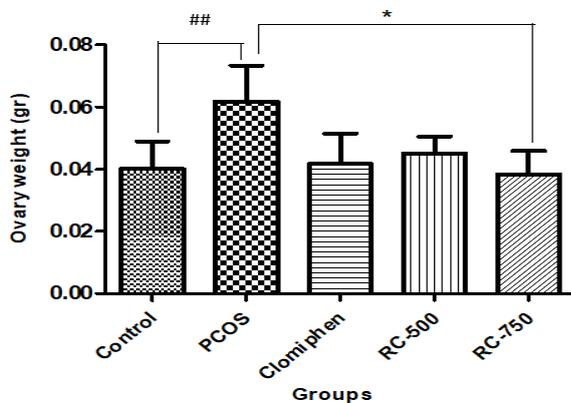
Treatment with red clover significantly reduced markers of oxidative stress such as MDA and NO and increased the level of potential antioxidant markers such as, GSH, CAT, and SOD in letrozole-induced PCOS model of rats. SOD is an antioxidant enzyme in live organisms. It protects the tissues and cell against damages caused by superoxide radicals. SOD reduces superoxide radicals generated by cell injuries via conversion of superoxide radicals into hydrogen peroxide (38). Superoxide scavenging capacity of plant extract may be mostly due to the presence of secondary phytochemicals such as total phenol and flavonoids. CAT enzyme hydrolyzes H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O and 1/2 O<sub>2</sub>. This enzyme has an important role in protecting DNA, proteins, and cellular lipids (39). NO is a very useful mediator in some physiological and biologic pathways in the human body. Its antioxidant and antitumor potentials were reported in the literature (40, 41). Deviation in GSH levels could be an early marker in the development of PCOS. GSH is an important antioxidant present in all the cells. The level of this non-enzymatic antioxidant parameter may be because of increased turnover, to prevent oxidative damage. It protects cells against free radicals, peroxides and other toxic compounds (42, 43). Furthermore, lipid peroxidation is mostly used as an indicator for oxidative tissue injury, which leads to cell necrosis and inflammation. Inflammation results in the production of reactive oxygen species. Therefore, the studies indicated that the incidence of

stereology study in PCOS group, the total volume of the ovary, cortex, medulla, and number of cysts were increased and a reduction is the number of oocytes compared with the control group. Treatment with red clover showed reduced volumes of ovarian, medulla



**Fig. 8.** Histological sections of rat ovary were stained with hematoxylin and eosin in different groups (20X magnification).

Control or Normal: saline recipient, PCOS; Letrozole recipient, RC-500: Red clover 500 mg/kg recipient; RC-750: Red clover 750 mg/kg recipient; PF: Primary Follicle; PAF: Pre antral Follicle; AF: Antral Follicle; GF: Graaf Follicle; CL: Corpus Luteum; OC: Oocyte. Letrozole-induced PCOS rats indicate large cystic follicles (CF); Red clover 500 mg/kg treated PCOS rats indicate well-defined thecal cell layer; Red clover 750 mg/kg treated PCOS rats indicate normal follicle.



**Fig. 7.** Effect of red clover treatment on ovary weight in letrozole induced PCOS rats.

Control; Normal saline recipient, PCOS; Letrozole recipient, RC500: Red clover 500 mg/kg; RC750: Red clover 750 mg/kg; Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple.

# denotes statistical significance in comparison to normal group rats.

\*denotes statistical significance in comparison to PCOS group rats. ##p<0.01; \*P<0.05.

PCOS was closely related to oxidative stress and chronic low grade inflammation (44, 45). In the present research, MDA is an indicator of lipid peroxidation that significantly increased in polycystic ovaries and red clover is able to restore it.

The ovarian weight in the PCOS group showed a significant increase compared to the normal group ( $p<0.05$ ) (Fig. 7). Consumption of clomiphene and red clover extracts significantly reduced ( $p<0.05$ ) ovarian weight compared to PCOS group (Fig. 7). Ovarian

and cortex. The number of cysts and number of oocytes are increased in respect of PCOS group.

The various histological studies revealed that a large number of cystic follicles were observed in PCOS group, however the cysts were not observed in other groups. Growth of ovarian follicles in the early stages did not occur. Their granulosa cells were destroyed and follicles became a cyst. Follicles were observed in the red clover treatment groups in different stages of growth (primary, preantral, antral and graphs) and corpus luteum that indicated ovulation (Fig. 8).

The volume of the cortex and total volume of the ovary were significantly reduced in the red clover groups as compared with the control rats. These results may be due to the absence of corpus luteum, reduction in the number of antral follicle, increase in the number of atretic follicles. Ovary atrophy occurs in the PCOS rats. Furthermore, red clover may induce oxidative stress and ovary apoptosis that can result in a reduction in the ovary volume.

Phytoestrogen has a chemical structural similarity or functional similarity to estrogens that resulted in similar hormonal activity, it further binds to estrogen receptors. Hence they have a protective effect against PCOS and hormone-dependent cancers. As isoflavones are major phenolic compounds found in addition to phytoestrogens in red clover, probably play important role of red clover in the prevention and treatment of PCOS (18).

## Conclusion

PCOS as a hormonal disorder common among women of reproductive age is one of the most common causes of female infertility. It has been reported that PCOS is a composite heterogeneous condition with multifactorial etiology like genetic, oxidative stress, and the environmental factors. The study indicated that red clover as a medicinal plant can be useful for treatment of PCOS similar to standard drug (clomiphene citrate). It is due to antioxidant properties of the plant that can be used as a potential source for production of natural anti-PCOS drugs with lower side effects.

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## Authors' contributions

AM and MJB designed the study. ZA, FB, and GZM contributed to carry out the experimental part of the work. All authors prepared the content of this manuscript and completed the final version.

## Conflict of interests

The authors declare that there are no conflicts of interest.

## Ethical issues

All procedures of the study were performed according to the approved local Animals Ethics Committee (Protocol approval number: 150/2014).

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