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The synergistic effect of *Paeonia spp and Glycyrrhiza glabra* on polycystic ovary induced in mice

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Abstract: *Paeonia spp* and *Glycyrrhiza glabra* were traditionally used to treat female hormonal problems. This study was conducted to investigate the effect of root extract of these plants on ovarian follicles after induction of polycystic ovary syndrome (PCOS). 25-day-old NMRI mice were divided into 4 groups: control, dehydroepiandrosterone (DHEA), and DHEA plus 50mg/kg and 100mg/kg plant extract. Animals in the DHEA group received a daily dose of 6mg/100 g.b.w dissolved in 0.05ml sesame oil for 20 consecutive days through subcutaneous injection (0.2ml); the control group received sesame oil and saline only through i.p; and the other two groups received herbal extracts through i.p. At the end, blood samples were taken to examine hormonal changes and isolate ovarian tissue. There was a significant difference in the level of testosterone and fasting insulin between the polycystic group and treatment groups (50 and 100mg/kg doses). The results showed a significant difference between the control and DHEA treated groups in terms of the number of graph and primary follicles ($P<0.05$). The results of this study indicated that the mixture of *paeonia spp* and *glycyrrhiza glabra* extracts with the administered dosage had positive effects on the status of follicles in the ovaries in polycystic ovary syndrome.

Keywords: Polycystic ovary syndrome, *Paeonia spp*, *glycyrrhiza glabra.*

INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most complex endocrine abnormalities that characterized by severe changes in endocrine hormones, and metabolic disorders. most patients, have a wide range of clinical symptoms, include: irregular menstruation, infertility, and symptoms associated with increased androgen (Norman *et al*., 2007), obesity, hyperinsulinism, hyperandrogenemia, insulin resistance. Hormonal disorders including elevated androgens, abnormal relative ratio of the gonadotropins *Luteinizing* hormone (LH) and follicle stimulating hormone (FSH) mediate the symptoms (Norman *et al*., 2007). Endocrine imbalances take place in the context of disordered ovarian folliculogenesis, chronic anovulation, clinical symptoms of hyperandrogenism and metabolic syndrome (Norman *et al*., 2007). Depending on fertility needs, the oral contraceptive pill (OCP) and ovulation induction with clomiphene citrate are among pharmaceutical treatments for menstrual irregularity (Group, 2008, Brown *et al*., 2009) metformin-mediated improvements *in insulin sensitivity.* But these drug have side effect such as clomiphene-induced ovulation has proven to be successful, pregnancy rates remain strangely low (Messinis, 2005). , metformin usage is associated with high incidence of adverse effects such as vomiting, nausea and gastrointestinal disturbances (Tanbo *et al*., 2018). Herbal medicines are complex treatments that have the potential for antagonistic and synergistic interactions between compounds (Nadu, 2018). Simultaneous interactions with different body systems, both biochemically and by changing organ function, can make the effects within the body more complex (Colalto, 2018). Among the most important plants in treating women's illnesses mentioned in many medical textbooks are *Paeonia spp* and *Glycyrrhiza glabra* that have traditionally been used to treat menstrual irregularities and to improve fertility. According to studies, *Paeonia spp* and *Glycyrrhiza glabra* extracts resolve hormonal imbalance especially by regulating the level of sex hormones (Jacobson *et al*., 2018). Studies have shown that root extracts of *Paeonia spp* and *Glycyrrhiza glabra* can increase estradiol by regulating gonadotropin secretion, decreasing reactive oxygen species (ROS) levels, producing ovarian androgens, decreasing cystic follicles, and changing the morphology of the ovary. Both Chinese and Western herbalists have used paeonia to treat gynecological conditions; Western herbalists use it for PCOS, hyperprolactinemia, ovarian failure, endometriosis and androgen excess. It has been demonstrated that white peony can positively affect low progesterone, reduce elevated androgens (testosterone) and act to modify estrogen and prolactin (Jacobson *et al*., 2018). In vitro experiments have shown that the active constituent

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Paeoniflorin can affect the ovarian follicle by acting on the aromatase enzyme (Ong, 2018). Aromatase has a significant role in follicle maturation, ovulation and corpus luteum function, synthesis of steroid hormone and regulating the conversion of androgens to estrogens. The biofeedback in the pituitary and hypothalamus depend on aromatase to regulate Gonadotropin-releasing hormone (*GnRH*) and prolactin (Wu and Ng, 2018). This study examines the synergistic effects of *Paeonia spp* and *Glycyrrhiza glabra* on PCOS treatment in an animal model.

MATERIALS AND METHODS

DHEA and sesame oil were bought from Merck (St. Louis, MO, USA), *Paeonia spp* and *Glycyrrhiza glabra* extracts were bought from Adonis Gol Darou (Iran, Tehran), and ELISA kit for evaluation testosterone was purchased from (Zell Bio, Germany), kit for fasting insulin was purchased from (Zell Bio, Germany) All of the chemicals used in Hematoxylin and eosin *(*H&E*)* staining were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Induction of PCOS model

25-day-old NMRI mice were divided into 4 groups: control, DHEA, and DHEA plus 50mg/kg and 100 mg/kg doses of plant extract. Animals in the DHEA group received a daily dose of 6mg/100g.b. w dissolved in 0.05 ml sesame oil for 20 consecutive days through subcutaneous injection (0.2ml); the control group received sesame oil and saline only through i.p; and the other treatment groups received mixed *Paeonia spp* and *Glycyrrhiza glabra* extracts through intraperitoneal (I.P). All mice were kept under controlled conditions with lighting and ad libitum access to food and water. During the treatment period, animals were weighted every two days, and vaginal smears were taken daily starting from 10 days after the first injection until the end of the experiment. All of the mice had remained in the estrus cycle. Blood samples were taken from the control group on the day of estrus. The ovarian tissue was isolated and placed in formalin 10% (Huang *et al*., 2015).

Blood sampling

The mice were anesthetized with ketamine and xylazine. Blood samples were taken from the heart and transferred to the laboratory to examine fasting insulin testosterone levels. At the end of treatment, blood samples were taken from the heart after 7 hours of fasting. The serum was isolated and kept at -80°C for hormonal examination. Fasting testosterone and insulin levels were measured and the results were recorded.

Morphology of the ovary

Immediately after the collection of blood samples, the ovaries were quickly isolated and fixed in formalin 10%. After insertion in paraffin and after usual histological methods, 5 µm sections were placed on a slide and stained with hematoxylin-eosin.

STATISTICAL ANALYSIS

Data are represented as the mean± SEM from at least three replicates per experiment. Statistical significance was determined as indicated; by one-way analysis of variance followed by Bonferroni, post hoc analysis, as appropriate using SPSS 19.0 software. A P value of<0.05 was considered statistically significant.

Ethical approval

All procedures involving mice were operated under strict criteria on the basis of the Guide for Care and Use of Laboratory Animals of Abadan University of medical science, and the moral code of this research (IR-ABDANUMS.1395.119) was also obtained from Abadan University of Medical Sciences.

RESULTS

Vaginal smear results

One day after DHEA injection, most mice showed vaginal smear with a mixture of cells. On the second day after injection, about two thirds of the animals showed estrus smear or a mixture of proestrus-estrus. In some mice, a mixture of proestrus-estrus was observed in this phase. No regular pattern of reproductive cycle was observed in the animals in this phase. During this phase, control mice continued their natural cycle (fig. 1). Weight change of animals in different groups did not differ significantly.

Ovarian histology

As illustrated in fig. 2, large cystic follicles with thin granulosum layer involving about 2 or 3 cell layers, a few primary follicles, which characterize PCOS, and a small number of corpora lutea were observed in ovaries treated with DHEA in adult mice. In the control group, unlike the treatment group, the ovaries lacked cysts and were full of primary follicles and corpus luteum, indicating normal ovulation (fig. 2).

After treatment of polycystic ovaries with a mixture of *Paeonia spp* and *Glycyrrhiza glabra* extracts, morphological studies showed that there were several cysts of different sizes at a lower dose (50mg/kg). There were no cysts in the ovaries treated with increasing dosage (100mg/kg doses); also, the number of corpora lutea that marked the beginning of ovulation increased in treated samples (fig. 2 and 3). In order to determine the effect of *Paeonia spp* and *glycyrrhiza glabra* extracts mixture on follicular development, follicles were divided into the following 5 groups based on their morphology: 1. primordial follicle; 2. primary follicle; 3. secondary follicle; 4. graff follicle; 5. corpus luteum.

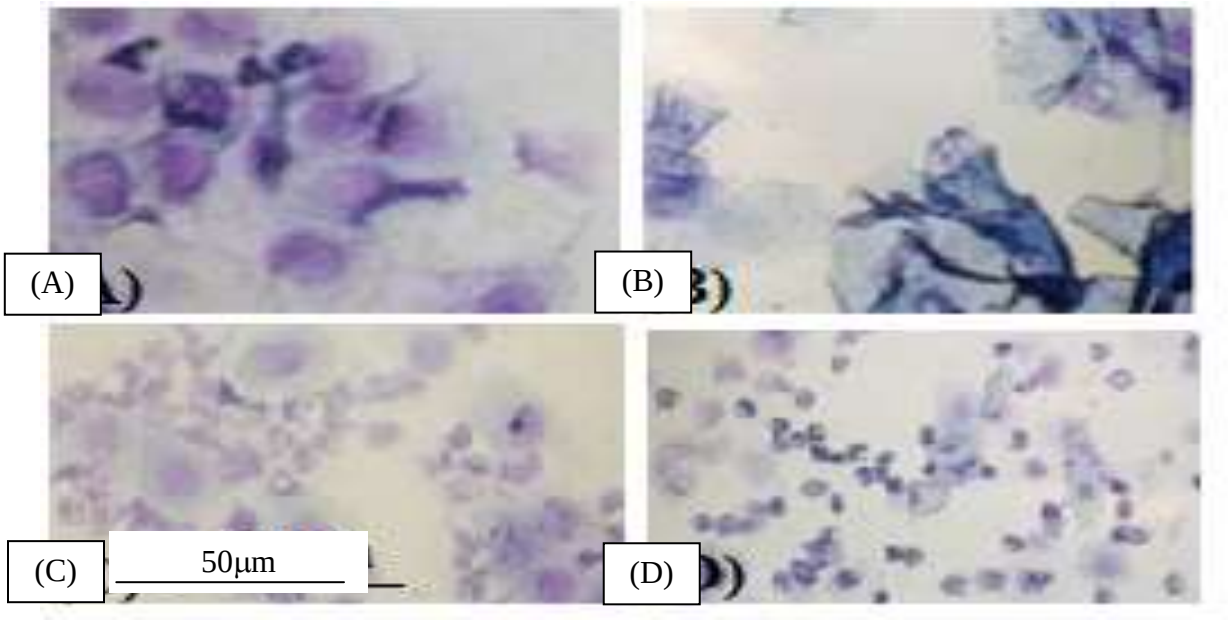

Fig. 1: Cytological assessment of vaginal smears can be used to identify estrous stage. (A) proestrus, (B) estrus, (C) metestrus, or (D) diestrus as described in representative results.

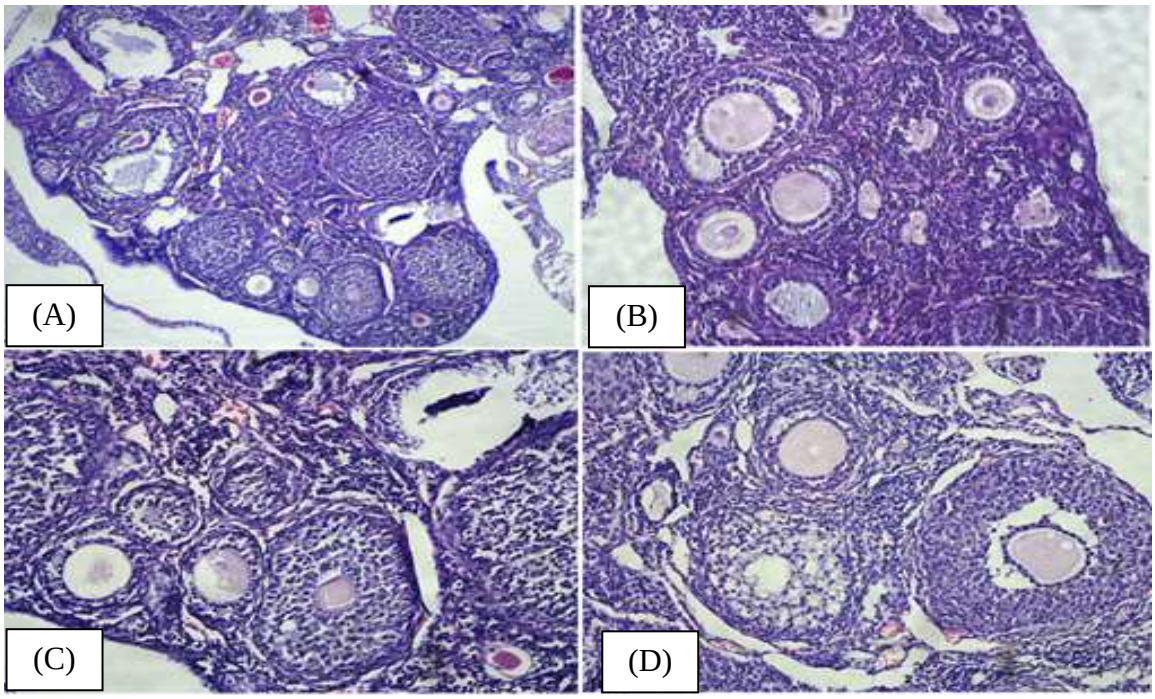

Fig. 2: Photomicrograph of the ovary tissue section with 40x magnification and hematoxylin-eosin staining (A = Control, B = PCOS, C = 50mg/kg drug, D = 100mg/kg drug)

The results showed a significant difference between the control and DHEA treated groups in terms of the number of graph and primary follicles ($P<0.05$). A number of large cystic follicles with a thin granulosum layer consisting of about two or three layers of cells were observed in the ovaries of the DHEA-treated group. In this group, a small number of corpora lutea were also observed, indicating ovulation.

In the control group, the ovaries lacked any cysts, and a large number of follicles were observed in different stages of follicular development; corpus luteum was also

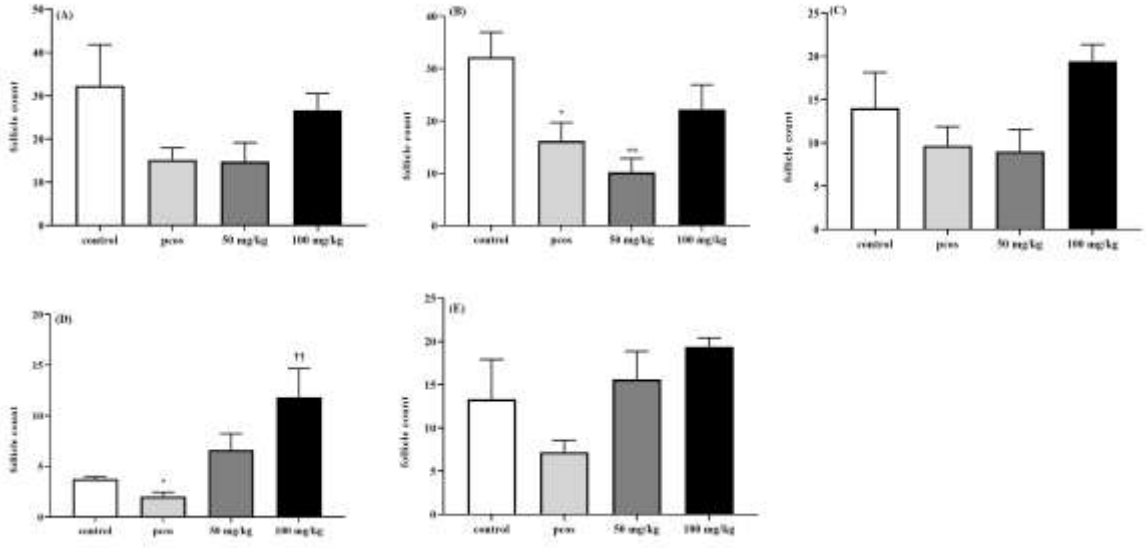

Fig. 3: Comparison of the mean number of follicles in the groups; (A = primordial follicle, B = primary follicle, C = secondary follicle, D = Graff follicle, E = corpus luteum). **P* < 0.05, ** *P* <0.01. *the comparison was made between groups *vs.* Control group, † the comparison was made between groups *vs.* PCOS group.

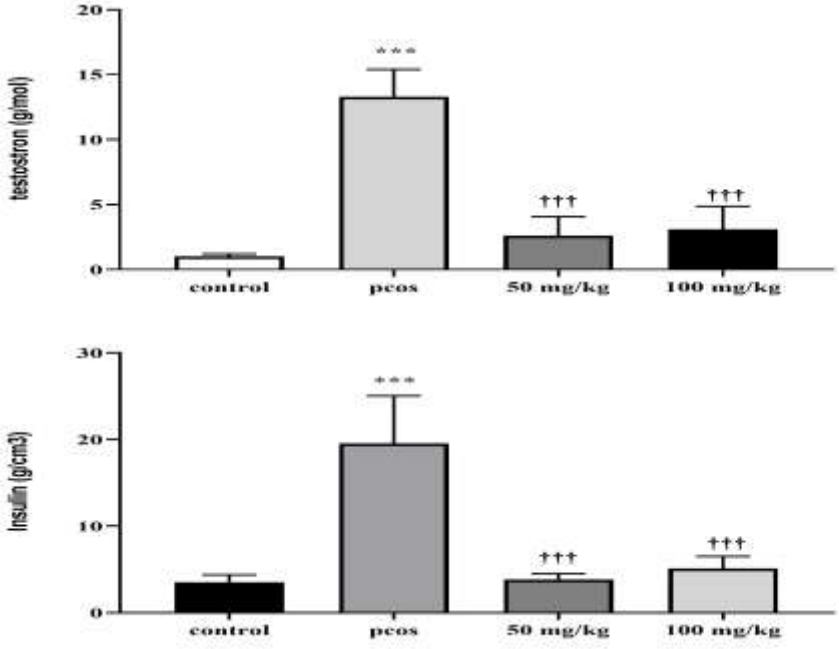

Fig. 4: Comparison of average levels of fasting insulin and testosterone in the groups. ****P* < 0.001. *the comparison was made between groups *vs.* Control group, † the comparison was made between groups *vs.* PCOS group.

observed. These results indicated the complete induction of PCOS phenotype in ovaries examined 20 days after DHEA treatment (fig. 2). PCOS mice were injected with 50 mg/kg and 50mg/kg doses of herbal extracts for 20 days and the control group received the same amount of sesame oil. After this period, the number of primary and graph follicles increased and the number of cysts decreased, and the changes were significant compared to the control group (fig. 3).

Evaluation blood serum level

According to fig. 4, changes in fasting insulin and testosterone levels in the polycystic and treatment groups (50 and 100mg/kg doses) showed a significant difference (p<0.001). Treatment with *Paeonia spp* and *Glycyrrhiza glabra* extracts was dose-dependent: higher doses resulted in lower levels of fasting insulin and testosterone.

DISCUSSION

In this study, the synergistic effect of *Paeonia spp* and *Glycyrrhiza glabra* root extracts was examined the ovarian follicles of PCOS mice. According to the results, the number of primordial, primary, secondary, graff and corpus luteum follicles in the PCOS group reduced significantly and the number of cystic follicles increased significantly compared to the control group. Research has shown that induction of PCOS by DHEA increases the number of cystic follicles. Changes in fasting insulin and testosterone levels have been observed. In previous studies, serum level changes were observed in the form of reduced FSH, progesterone, and increased LH, estradiol, and testosterone (Tamaya, 1986). A study was conducted on the effect of PCOS on ovarian follicles in mice. The results showed an increase in the percentage of cystic follicles in the PCOS experimental group (Gonzalez, 1999). The results of histological studies indicate morphological changes in polycystic ovary in the form of increased cystic follicles and reduced follicular groups and corpus luteum in DHEA treated mice. The mean percentage of preantral, antral, and corpus luteum follicles in the PCOS group decreased significantly and the percentage of cystic follicles increased significantly compared with the control group (Jones, 1980). Insulin, *Insulin-like growth factor 1* (IGF-1) and, *Insulin-like growth factor 2* (IGF-2) are among autocrine and paracrine regulators of the theca-interstitial (T-I) cells in humans and mice that can stimulate the proliferation and steroidogenic activity of these cells. Insulin and insulin-like factors increase the synthesis of androgens in theca and granulosa cells; this hormone also increases the free available testosterone in the bloodstream. Increased production of androgens ultimately causes follicular growth, resulting in an increase in LH level, which in turn increases body weight and ultimately reduces follicular atresia (Trounson, 2001). The results of this study indicated that the use of DHEA to induce PCOS significantly increased the testosterone levels in the serum of PCOS mice compared with the control group. On the other hand, the testosterone level in the experimental group decreased significantly after receiving *Glycyrrhiza glabra* extract, as compared to the PCOS group. This is consistent with the results obtained by Sakamoto and Takashashi (Sakamoto, 1988). Follicular androstenedione/ estradiol ratio is high in PCOS patients due to aromatase deficiency and mutation of p450 aromatase gene (Zheng *et al*., 2018). In polycystic ovaries, high levels of androstenedione are secreted from the single internal layer, which increases when it transforms to testosterone, while in PCOS women, part of the testosterone is associated with the adrenal gland (Aaltonen, 1999). In a study by Susan Arentz *et al.* (2014), it was shown that *Paeonia spp* reduced testosterone and *Glycyrrhiza glabra* reduced LH, prolactin, fasting insulin, and testosterone and increased corpus luteum in the PCOS model (Arentz S, 2014). In this study, synergistic effects of these two plants improved ovarian tissue and reduced testosterone and fasting insulin compared to non-mixed studies (studies that did not use *Paeonia spp*. and *Glycyrrhiza glabra* extracts together). Oxidative stress in PCOS patients has been shown to play an important role in follicular structure changes and overgrowth of ovarian mesenchymal tissue. Evidence suggests that free radicals play a decisive role in fertilization, especially in cases such as oocyte maturity formation and reduction of corpus luteum, ovulation and pregnancy (Batth, 2000). By increasing the activity of antioxidant enzymes, antioxidants can reduce oxidative stress in the ovarian tissue. It has also been shown that the use of antioxidants can significantly reduce the number of cystic follicles (Guérin, 2001). Another study suggested that, with their anti-inflammatory effects, *Glycyrrhiza glabra* compounds could potentially affect the serum level of sex hormones and trigger re-ovulation in PCOS mice (Movérare-Skrtic, 2006). In traditional medicine, *paeonia spp* is used to treat bacterial and fungal diseases; it has been effective in the treatment of bacterial diseases due to its antioxidant properties (Pahuja, 2012). Gonazales *et al*. reported that the level of follicular free radicals in PCOS people is more than in healthy individuals (Gonzalez, 1999). Therefore, free radicals in the follicular fluid seem to be one of the factors of non-ovulation in people with this syndrome (Gautam N.Allahbadia, 2011). Due to their antioxidant properties, *Paeonia spp* and *Glycyrrhiza glabra* reduce the level of free radicals in PCOS individuals (Tamaya, 1986), which subsequently leads to lower testosterone and estrogen levels. As a result, it can be suggested to use *Paeonia spp* and *Glycyrrhiza glabra* to treat ovarian disorders, including PCOS, and to increase the success rate of *in vitro* fertilization (IVF). A number of clinical trials have focused on the traditional Chinese/Kanpo formula known as Shakuyaku-Kanzo-To or TJ-68, which is a decoction of *Glycyrrhiza glabra* and *Paeonia lactiflora*, all of which show activity in the hormonal regulation of androgens. In one of these trial conducting on eight women with hyperandrogenism and oligomenorrhea, the formula was given for 2 to 8 weeks. This combination regulated the LH/FSH ratio. Over this period, the level of serum testosterone declined to less than 50ng/dL, leading to regular ovulation in seven of the eight women (Yaginuma T, 1982). In this study, a mixture of *Glycyrrhiza glabra* and *Paeonia spp* proved to reduce elevated testosterone level and induce ovulation.

CONCLUSION

Reduced tissular symptoms of PCOS mice by mixed *Paeonia spp* and *Glycyrrhiza glabra* extracts is probably due to the anti-inflammatory and antioxidant properties of this herbal product. It seems that reduced testosterone level, reduced thickness of the follicular sheath layer as well as increased number of corpora lutea in the ovaries treated with a mixture of *Paeonia spp* and *Glycyrrhiza glabra* extracts in a dose-dependent manner is an

evidence that suggests increased fertility in PCOS individuals. According to the results, it can be concluded that, with its antioxidant and protective effects, the mixture of *Paeonia spp* and *Glycyrrhiza glabra* extracts improves the status of follicles and decreases the tissular symptoms of PCOS patients.

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