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Yarrow (Achillea millefolium L.) Extract Produces Beneficial effects on Reproductive Parameters in Estradiol Valerate-Induced Polycystic Ovarian Syndrome in Rats

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Original Article

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Abstract:

Background: Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders with a spread of around 10% in the world. Yarrow (Achillea millefolium L.) is a medicinal plant with essential oils, steroids, flavonoids and phenolic compounds. The aim of this study was to investigate the effects of yarrow extract on blood hormones and ovarian histology in rat model of PCOS.

Methods: Seventy rats were equally allocated into seven groups: control; control+1.2g/kg body weight (BW) yarrow extract; PCOS, induced by single-dose intramuscular injection of estradiol valerate (EV, 1 mg/100g BW); PCOS+2 mg/kg BW clomiphene citrate (CC); PCOS+1.2, 2.5 and 5g/kg BW of yarrow extract for a period of 30 days. Vaginal smear was performed for 60 days after the EV injection. Hormone assays for LH, FSH, progesterone, testosterone and estradiol, and ovarian histology were also performed.

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Results: CC could moderate serum levels of FSH, estrogen and progesterone and concentrations of LH, LH/FSH and testosterone became normal, whereas CC did not have a significant effect on body and ovary weights, and number of ovarian cysts. Yarrow extract could ameliorate the body and ovary weights, cystic follicles and sex hormones in PCOS induced by EV in a dose-dependent manner. High dose of yarrow extract improved the detrimental effects of PCOS on body and ovary weights, and number of ovarian cysts, and caused them to reach normal levels.

Conclusion: Yarrow extract in 5mg/kg BW may be used as a nutritional supplement for adjunct therapy of PCOS patients.

Keywords: *Achillea millefolium* L, estradiol valerate, estrous cycles; ovary, sex hormone

INTRODUCTION

Polycystic ovary syndrome (PCOS), one of the most common endocrine disorders, is the reason for irregular menstruation and anovulatory infertility of women during reproductive life. This syndrome introduced as a menstrual dysfunction, hyperandrogenism, and metabolic disorder (1).

Several morphological events including increased thickness of ovarian cortex, presence of multiple follicular cysts and stromal hyperplasia occur in PCOS, which lead to interruption of folliculogenesis. Abnormal hormone levels of PCOS in serum are identified as an elevation in the androgenic hormones such as testosterone, androstenedione, and dehydroandrostenedione (2). Furthermore, sharper rise of LH compared to FSH is associated with hirsutism, oligomenorrhea, and amenorrhea (3). Subsequently, several abnormalities occur due to this syndrome including visceral obesity, increased body mass, insulin resistance and cardiovascular disorder (4).

Various experimental models have been developed to induce PCOS in rodents. Administration of rodents with dehydroepiandrosterone, antibodies to luteinizing hormone releasing hormone, letrozole, constantly light condition and estradiol valerate (EV) could induce PCOS (1, 5-8). A single dose, intramuscular injection of EV in 8-week old rats induced characteristics of human PCOS, such as appearance of large cystic follicles in the ovaries and change concentration of luteinizing hormone (LH) (8).

Despite widespread acceptance of clomiphene citrate (CC), an antiestrogen agent, as the first line medication for ovulation therapy in women with PCOS, some women show anovulatory cycle. This medication improves only 60–85% of anovulatory cycle cases (9). Furthermore, the rate of conception (40–50%) with CC is less than ovulation rate (10, 11). Thus, developing other alternative medicines is needed to treat PCOS. Medicinal plants with antioxidant properties have beneficial impacts on female reproductive systems (12). Therefore, traditional remedies and medicinal plants with minimum side effects could

be used as alternative drugs for PCOS (13, 14).

Yarrow (*Achillea millefolium L.*) is one of the oldest medicinal plants (for over 3000 years) and is widely distributed across the world (15). This ancient herb has noticeable antibacterial, antifungal, antiparasitic, anti-inflammatoy, hemostyptic, antioxidant, antispasmodic, estrogenic, anticancer, gastroprotective and hepatoprotective activities due to containing essential oils, steroids, flavonoids and phenolic compounds (16). Boswell-Ruys et al. (2003) reported that yarrow extract was safe for reproduction without contraceptive, abortifacient, and teratogenic activities (17). As well, Dalsenter et al. (2004) reported that oral gavage of yarrow extract for 90 days did not cause any reproductive toxicity in male rats (18). Thus, given numerous benefits of yarrow extract without any toxicities on the reproductive system, the aim of this study was to investigate the effects of ethanolic extract of yarrow on ovarian histology and blood serum hormones in rat model of estradiol valerate (EV)-induced PCOS.

MATERIALS AND METHODS

Animals and Experiment Design

Seventy Wistar albino virgin female rats (8 weeks old) weighting 180-250 g were equally allocated into seven groups (n=10). Every very five rats were housed in a cage under a 12:12 hour light: dark cycle at rooms temperature (22 ± 2°C) and relative humidity (50±5%), and ad libitum access to food and water. All animal care and experimental procedures were approved by the Ethics Committee of AJA University of Medical Sciences (IR.AJAUMS.REC.1399.015).

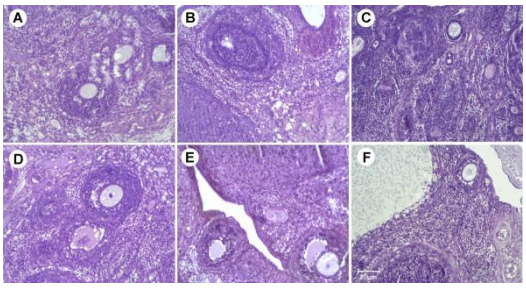

Fig. 1. Sections of ovaries from control (A), estradiol valerate treated (B), PCOs+clomiphene citrate (C), and PCOs+1.2, 2.5 or 5 g/kg BW of yarrow extract groups (D-F). Slides were stained with hematoxylin and eosin, scale bar = 20 µm.

Twenty rats served as a control (C) and Control+1.2 g yarrow extract per BW (C+1.2Y) and did not receive any injection (10 rats for each group). Fifty rats assigned to the PCOS groups and received a single dose, intramuscular injection of 1 mg/100g body weight (BW) of estradiol valerate (EV; Aburaihan Co., Iran) per kg BW of rats. All groups were evaluated by vaginal smear tests for 60 days after the injection, and then subdivided to four groups: one group did not receive yarrow extract (PCOS), one group received 2 mg/kg BW clomiphene citrate (PCOS+CC), and three other groups were gavaged daily at different doses (1.2, 2.5 and 5 g/kg BW) of yarrow extract for a period of 30 days (19). All rats were weighed in the first and last days of the experiment.

Table 1: Effects of Yarrow extract on body and ovary weights in EV induced PCOs rats

Groups	Body weight (g)		Ovary weight (mg)
	First experiment day	Last experiment day	
Control	193.87±3.97	236.50±10.23[b]	123.20±4.63[b]
C+1.2Y	197.23±4.01	226.50±7.63[b]	121.36±6.48[b]
PCOs	196.46±4.87	295.66±8.90[a]	134.44±5.72[a]
PCOs+ CC	198.06±3.91	281.32±9.56[a]	132.21±4.39[a]
PCOs+1.2Y	201.23±4.63	276.33±7.35[a]	133.75±5.12[a]
PCOs+2.5Y	194.71 ±4.41	256.50±6.02[a]	130.75±4.43[a]
PCOs+5Y	199.59±5.06	240.33±3.51[b]	124.30±3.23[a]

Control+1.2 g yarrow extract (C+1.2Y), estradiol valerate treated (PCOs), PCOs+clomiphene citrate (PCOs+CC), and PCOs+1.2, 2.5 or 5 g/kg BW of yarrow extract groups (PCOs+1.2Y, PCOs+2.5Y and PCOs+5Y). Values are means ± SEM. Different letters show significant differences among the groups ($P < 0.05$).

Table 2. Number of primordial, primary, secondary, mature and atretic follicles, corpus luteum and cyst

	Primordial follicle	Primary follicle	Secondary follicle	Mature follicle	Corpus luteum	Atretic follicle	Cyst
Control	55.0±3.7	28.0±2.3	21.0±2.0 [ab]	7.2±0.6	3.3±0.3	5.7±0.7	0.0±0.0
C+1.2Y	57.0±3.5	33.0±3.4	25.0±2.2 [b]	7.4±0.7	4.0±0.6	6.9±0.9	0.0±0.0
PCOs	50.0 ±3.6	24.0±2.3	17.0±1.5 [a]	5.0±0.5	2.5±0.3	7.0±0.8	9.0±0.6[a]
PCOs+ CC	50.0±3.7	27.0±2.3	19.0±1.4 [ab]	5.8±0.8	2.7±0.2	5.5±0.6	8.5±0.9 [ab]
PCOs+1.2Y	51.0±3.7	25.0±2.4	17.0±1.4 [a]	5.2±0.8	3.6±0.4	5.5±0.7	8.5±0.7 [ab]
PCOs+2.5Y	52.0±3.4	23.0±2.1	15.0±1.3 [a]	5.1±0.8	3.2±0.3	5.2±0.8	8.7±0.7 [ab]
PCOs+5Y	53.1±3.4	29.0±2.3	19.0±1.3 [ab]	7.0±0.6	3.0±0.3	5.5±0.9	6.0±0.7 [b]

[a-b]Means in a column with different letters are significant different ($P < 0.05$).

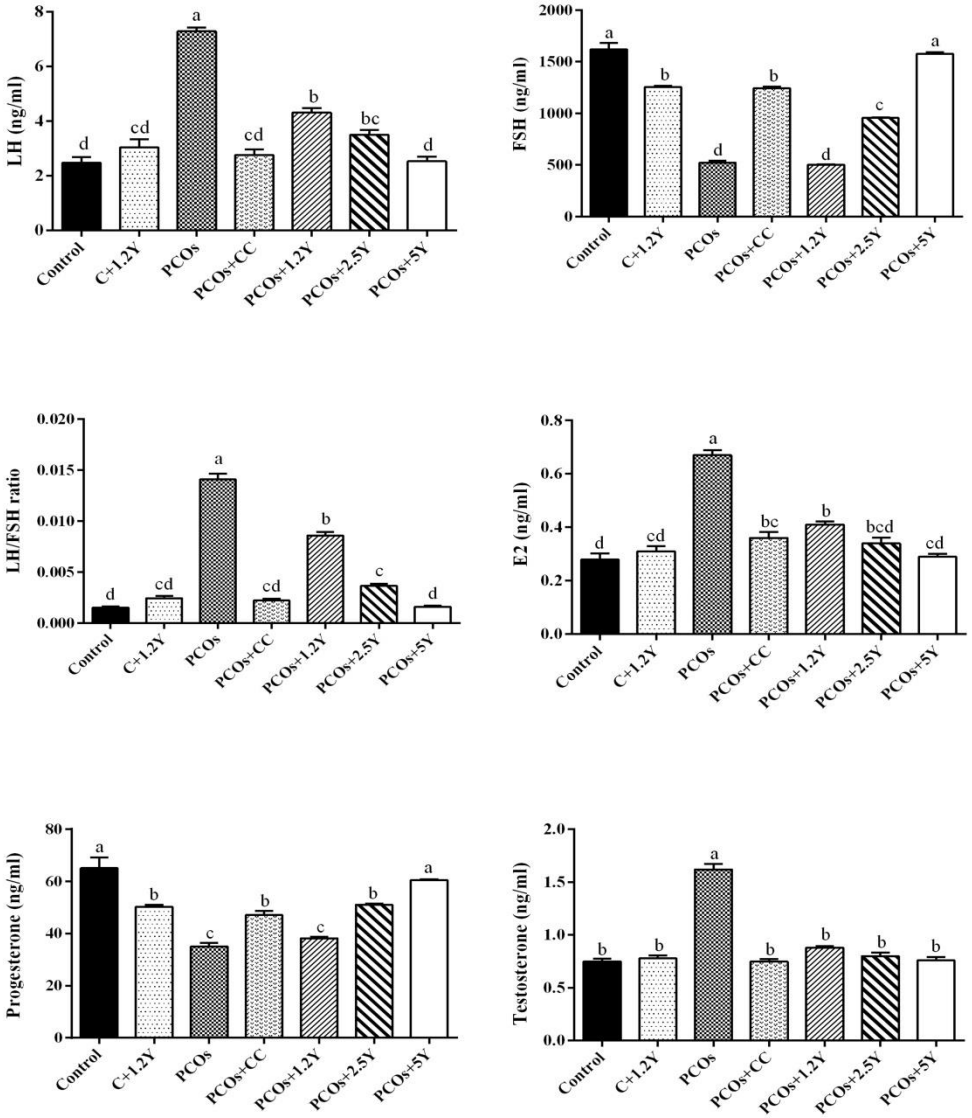

Fig. 2. Effects of different concentrations of yarrow extract on serum hormone levels in the rats treated by estradiol valerate. Luteal hormone (LH), follicle stimulating hormone (FSH), LH/FSH ratio, estradiol (E2), progesterone and testosterone were measured in control, Control+1.2 g yarrow extract (C+1.2Y), estradiol valerate treated (PCOs), PCOs+clomiphene citrate (PCOs+CC), and PCOs+1.2, 2.5 or 5 g/kg BW of yarrow extract groups (PCOs+1.2Y, PCOs+2.5Y and PCOs+5Y). Values are means ± SEM. Different letters show significant differences among the groups ($P < 0.001$).

Extraction of *Achillea millefolium L.*

600 grams of dried powdered yarrow (Achillea millefolium L.) were macerated with ethanol 70 (3 L) for 12 h in a dark room. The solvent extraction was repeated thrice. After filtering through a Whatman filter paper, the extract was concentrated to dryness on a rotary evaporator and weighed. The percentage yield of the given extract was calculated by dividing the weight of yarrow extract by the weight of dried powdered yarrow. All extracts were dissolved in normal saline solution to make the required concentration and then were used throughout treatment processes (20).

Vaginal smears

Estrous cycle was evaluated by vaginal smears under a light microscope for the relative frequency of nucleated cornified and epithelial cells, and leukocytes. Briefly, vaginal lavage was done with normal saline and small drops put on a glass slide. After air-drying, the glass slides were stained with 0.1% toluidine blue solution at room temperature for 2 seconds. The regular rats estrous cycle usually persists for about 4 days. The present of cornified cells in the vaginal smears for a minimum of 10 consecutive days was considered as persistent vaginal cornification (PVC) and represents the induction of PCOS (21).

Hormone assays

At the completion of the experiment, rats were anesthetized with ketamine and xylazine. Blood was collected by heart puncture, and serum samples were isolated by centrifugation at 6,000 rpm for five minutes. Serum levels of luteinizing hormone (LH), follicular stimulation hormone (FSH), progesterone (P4), testosterone (T), and estradiol (E2) levels were determined by ELISA kits Biocompare (South San Francisco, CA, USA) according to the manufacturer's protocol.

Ovarian histology

Ovarian samples for follicle classification were collected and fixed at least for 24 h in formalin 10%. After tissue processing, they were embedded in paraffin, and serially sectioned in 5 μm, and mounted on glass slides. Serial ovarian sections were then stained by hematoxylin & eosin and observed under a light microscope (Olympus, Japan) with 40× and ×100 magnifications (Fig. 1).

Follicles were defined based on morphology as follows: primordial: a follicle with a single layer of flattened granulosa cells; primary: a follicle surrounded by a single layer of cubidal granulosa cells; secondary: a follicle with at least two layers of cubidal granulosa cells without antral space (only follicles with a visible nucleolus were counted.); antral: a follicle with single large antral space; atretic: a degenerated follicle or follicle with pyknotic nuclei in granulosa cells;

corpora lutea and cystic follicles (22, 23). All of these types of follicles were counted on five ovarian sections per rat.

Data Analysis

Assumptions of normality and homogeneity of variance were investigated. Final BW was analyzed by ANCOVA. Parametric data were analyzed using one-way ANOVA, and Tukey was used for group comparison. Nonparametric data were analyzed by Kruskal-Wallis test followed by Dunn's test to detect the specific differences among groups. Data were expressed as means ± standard error of measurement (SEm). $P < 0.05$ was considered significance level. All graphs and statistical tests were performed using the GraphPad Prism version 6 (CA, USA).

RESULTS

Body and ovary weights

Data on body and ovary weights are presented in Table 1. In PCOS group, body and ovary weights increased when compared to control group ($P<0.05$). CC treatment or using 1.2 and 2.5 g/kg yarrow extract had no significant effect on body and ovary weights, whereas 5 g/kg yarrow extract significantly decreased body and ovary weights in PCOS rats ($P<0.05$), which was not significantly different from that in the control group.

Estrous cycles

Forty rats were assigned to the PCOS groups by administration of EV on the estrus. After two days, most of the female rats were in estrus. Irregular cycles appeared within one week after EV injection and most of the animals showed PVC by three weeks after EV treatment. Treatment of rats with 2.5 or 5 g/kg BW yarrow or 2 mg/kg BW clomiphene citrate improved most disorders of estrous cycle in EV-treated rats, however most of them in PCOS and PCOS+1.2Y groups continued to irregular cycles. All animals in control and C+1.2Y groups exhibited regular cycles during the whole experience.

Ovarian follicle numbers

In this study, the number of primordial, primary, secondary, mature and atretic follicles as well as number of corpus luteum and cysts was counted in the ovaries of all groups. Follicles in various stages of development were observed in the all ovaries groups. Results showed no significant differences among the groups with respect to primordial, primary, mature and atretic follicles and corpus luteum ($P>0.05$). The number of secondary follicles in the PCOS group (17.0±1.5) was significantly lower than that in C+1.2Y (25.0±2.2, $P<0.05$). Number of cysts in ovaries increased in the PCOS group (9.0±0.6) and significantly improved in rats given 5 g/kg BW yarrow (6.0±0.7, $P<0.05$, Table 2).

Serum hormone levels

Ovarian structure and function is predominantly controlled by the levels of hormones in blood serum. Thus, levels of FSH, LH, E2, T and P were measured in the serum blood of the rats. The levels of LH, LH/FSH ratio, E2 and T significantly increased after inducing PCOS in rats through EV treatment ($P < 0.001$). Serum FSH and P levels also decreased after administration of EV ($P < 0.001$). The levels of LH, LH/FSH ratio and T in the clomiphene citrate group were similar to those in the control group. However, other serum hormones partially improved after clomiphene citrate treatment. Our results indicated that 1.2 and 2.5 g/kg yarrow extract incompletely moderated the adverse effects of EV treatment on LH, FSH (not for 1.2 g/kg), LH/FSH ratio, E2 and P in blood serum. However, the serum level of T in both concentrations was equal to that in the control group. High dose (5 g/kg) of yarrow extract completely improved all adverse effects of EV on the studied serum hormones (Fig. 2).

Discussion

EV effectively induced PCOS symptoms such as increase in the body and ovary weights with irregular estrous cycles and PVC after one and three weeks of EV injection, respectively, and ovaries were cystic. Serm levels of FSH and P decreased, however the levels of LH, LH/FSH ratio, E2 and T increased after EV injection. The effect of yarrow extract on sex hormone parameters in PCOS rats was dose-dependent, so that with increasing the extract concentration, the adverse effects of the EV were improved in the studied blood parameters. Low and moderate doses of the extract could not improve adverse effects of PCOS on body and ovary weights, and number of ovarian cysts, while high dose of the extract ameliorated all studied parameters, and reached them to normal level. Thus, yarrow extract could improve the body and ovary weights, cystic follicles and sex hormones in PCOS induced by EV in a dose-dependent manner; however, the highest dose of yarrow extract was more effective than CC treatment.

Increase in body and ovarian weight after EV treatment may be related to the aggregation of fatty tissue in the body and creation of follicular cysts with higher follicular fluid in these cystic follicles. The results of another study showed that PCOS was associated with obesity (24). In PCOS women, the levels of LH, E2, and T are higher and levels of FSH and P are lower than the normal women (25). Our results were consistent with a study that showed that EV injection induced clinical parameters of PCOS in rat (26). Changes in serum levels of these hormones may be related to depletion of aromatase enzyme in PCOS ovaries that leads to an increase in the androgens content. Moreover, androgens, through increasing the FSH receptors, cause a depletion in the serum FSH level and a promotion of the LH hormone (27).

Given these results, a high number of cystic follicles may be related to aggregation of follicular fluid in the cystic follicles and high levels of E2 in follicular fluid (28). It has been demonstrated that increase in androgens is associated with imbalance of antioxidants and oxidants, which finally leads to induction of PCOS (29).

Yarrow extract did not show any deleterious effects of body and ovary weights, histological and pathological parameters and hormones assay, which is in accordance with Dalsenter et al. study, who reported that no detrimental clinical effects were detected on reproductive systems of adult male rats. They also reported that estrogenic and antiestrogenic properties of this extract did not indicate any uterotrophic effects on immature female rats (18).

Similarity of CC to E2 structure helps it to bind to E2 receptors, and by alleviating the performance of E2 and promoting the function of P (30), improved several devastating effects of EV injections on serum FSH, E2 and P levels and concentrations of LH, LH/FSH and T, whereas it did not significant affect body and ovary weights, and number of ovarian cysts.

The major components (approximately 60% of total oil) in yarrow extract are eucalyptol, camphor, α-terpineol, β-pinene, and borneol. These components can diminish the diphenylpicrylhydrazyl radical and serve as a hydroxyl radical scavenger. Yarrow extract, in addition to producing antioxidant effect, inhibits the non-enzymatic lipid peroxidation (18). Accumulating evidence indicates a direct correlation between the level of oxidative stress and obesity in the PCOS women (31). Furthermore, reactive oxygen species (ROS) play a pivotal role in controlling various reproductive functions. The overproduction of ROS that occurs during PCOS could be moderated by antioxidant activity of yarrow extract (32). Another study indicated that yarrow extract ameliorated the level of fasting plasma glucose, reduced hypercholesterolemia and promoted of insulin sensitivity in obese rats. They concluded that the extract could be used as a nutritional supplement in obesity (33). Yarrow also reduced the expression of the lipogenic master regulator Srebf1 (34). This data suggested that yarrow may be responsible for reduction of fatty formation by decreasing metabolic stress due to its antioxidant capacity or downregulating lipogenic genes.

Flavonoids and polyphenolic acids, as main polyphenol compounds of yarrow (35), prevent the ovarian estrogenic activity by occupying the estrogen receptors (36) and alleviate the testosterone level by stimulating aromatase activity (37). Accumulating evidence indicates that plants with antioxidant activity and antiandrogenic effect, such as ginger, chamomile and green tea extract, can improve the adverse signs of PCOS in the ovarian

tissue and sex hormones (38-40), which is in agreement with our results.

CONCLUSION

Our result indicated that yarrow extract could improve the detrimental effects of PCOS on reproductive parameters including estrous cycles, ovaries morphology and sex hormones in mature female rats in a dose-dependent manner. Yarrow extract in 5 mg/kg BW may be used as a nutritional supplement for treatment of PCOS patients. Further studies are recommended to elucidate the effects of yarrow extract on women with PCOS.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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