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Original Research Article

Vitex agnus-castus extract improved the endocrine profile and important gene expression in rat ovaries with polycystic ovary syndrome

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Abstract

Objective: The beneficial effect of *Vitex agnus-castus* on the female endocrine system has been reported. This study examines the impact of *Vitex* extract (VE) on the serum anti-Mullerian hormone (AMH), sex hormones, and *$P450_{arom}$*, *AMH*, and *CTRP6* gene expression in the ovaries of a rat model of PCOS.
Materials and Methods: Thirty-two rats with regular estrous cycles were enrolled as controls, polycystic ovary syndrome (PCOS), and two treatment groups. PCOS was induced by estradiol valerate. The treatment groups (T1 &T2) received VE (150 or 200 mg/kg body weight, respectively) for 30 days following PCOS induction. Serum and left ovary were used to assess hormone levels and *$P450_{arom}$*, *AMH*, and *CTRP6* gene expression.
Results: In the T2 group, a significant decrease in estradiol (E2, testosterone, and AMH levels (p=0.028) was observed with no significant change in DHEA (p=0.967) in comparison with the PCOS group. AMH in the serum was significantly higher in the PCOS group than in all the other groups (p=0.021). The level of progesterone was not considerably changed between treatments and the PCOS group (p=0.11). The expression of *$P450_{arom}$* in the T2 group was significantly higher than the PCOS group (p=0.003) and had no significant difference from the control (p=0.200). The expression of *AMH* (p=0.003) and *CTRP6* was significantly (p=0.0001) higher in the ovaries of the PCOS group compared to other groups, and consumption of the extract improved the evaluated parameters significantly.
Conclusion: The endocrine profile and gene expression were improved in the rat ovary following administration of VE, and therefore, the therapeutic effect of VE may be expected in PCOS.

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Introduction

Polycystic ovary syndrome (PCOS), which is a chronic, complex, heterogeneous disorder, is an important cause of ovulatory and menstrual irregularity, subfertility, and infertility in women. It is identified as one of the most widespread female endocrine disorders in reproductive age (Azziz et al. 2016). PCOS comprises metabolic, endocrine, reproductive, and psychological characteristics, however, the cause of this syndrome is not well understood (Rodriguez Paris and Bertoldo 2019). Depending on the definition and the population studied, the prevalence is commonly regarded as 6-20% (Azziz et al. 2016). It has been found that uncontrolled androgen production is a basic feature of PCOS (Franks 2021). People with hyperandrogenic PCOS have elevated levels of several androgens, including testosterone, androstenedione, and dehydroepiandrosterone sulfate (DHEAS) (Keefe et al. 2014).

A deficiency in aromatase activity is proposed as a reasonable cause of intra-ovarian disorder in steroidogenesis and PCOS development (Rajan and Balaji 2017). Deficiencies in hormone regulation and intracellular signaling for *CYP19A1* induction, inadequate levels of follicle-stimulating hormone(FSH), and epigenetic changes in regulatory regions of this gene are among the major causes of aromatase deficiency and the pathogenesis of PCOS (Yu et al. 2013). Few phenomena such as small levels of $P450_{arom}$ mRNA, reduced ovarian aromatase activity, and estrogen production in different sizes of follicles in PCOS has been reported in earlier studies (Chen et al. 2015).

Anti-Mullerian hormone (AMH) as a member of the transforming growth factor-beta family is a glycoprotein (Dumont et al. 2015). New studies have focused on both intra-ovarian and central activities of AMH in the mechanism of anovulation and the etiology of PCOS (Franks and Hardy 2020). In PCOS, an increase in the number of ovarian follicles at any development stages was remarkable, especially in the pre-antral and small antral follicles (Dewailly et al. 2020; Maciel et al. 2004). These follicles are the ones that primarily produce AMH (Bhide et al. 2015). AMH levels are 2-4 times higher in women with PCOS than in healthy women. (Dewailly et al. 2020). Elevated AMH levels restrain FSH-stimulated follicular activation and growth, resulting in the arrest of follicles (Krishna 2019). Moreover, the AMH inhibits FSH-induced aromatase production, which probably contributes to hyperandrogenism (Homburg 2009). The paracrine effect of AMH on theca cells results in enhanced androgen production (Krishna 2019). Recent experimental data have shown that AMH probably has extra-gonadal effects; importantly, it increases the action of GnRH neurons. Numerous *in vitro* and *in vivo* investigations revealed that AMH improved GnRH-dependent pulsatile luteinizing hormone (LH) secretion via a central act. In addition, , AMH appears to exploit its effect by regulating the activity of gonadotropic cells in the pituitary (Dewailly et al. 2020). AMH reflects the primordial oocyte pool and its levels are associated with antral follicle number and ovarian volume (Pigny et al. 2006). Studies demonstrated that AMH level is associated with testosterone, different phenotypes of follicles, and the severity of PCOS (Jacob et al. 2017). This led to the proposal that AMH is a biomarker for polycystic ovarian morphology (PCOM) and could be utilized in the diagnosis of PCOS (Dabadghao 2019).

Recent studies have shown a functional role for *CTRP6* in the regulation of follicular development, oocyte maturation, ovulation, corpus luteum function, steroidogenesis, angiogenesis, regulation of cell cycle, apoptosis, and insulin metabolism. These roles are crucial for the normal functioning of oocytes (Wan et al. 2019). An increase in the expression of this gene can impair fertilization and implantation (Yin et al. 2019).

Increased expression of *CTRP6* gene following induction of PCOS has been reported in the animal model of PCOS, and treatment with vitamin D has been reported to attenuate the expression of this gene (Hakimpour et al. 2022). Increased serum levels of CTRP6 were also reported in women patients with PCOS, and it was proposed to be related to impaired glucose metabolism, as a major cause of PCOS (Sadeghi et al. 2020).

Various treatments are now accessible for women with PCOS, but their overall effect on fertility is under investigation. However, a few approaches are currently available, including lifestyle changes, hormone modulators, and laparoscopic ovarian surgery. (Lee et al. 2020). There is an increasing trend in the consumption of natural resources or plants for treating diseases in various societies and a wide range of commercial pharmaceutical products have been manufactured.

Approximately 25% of the world's approved drugs are derived from plants (Alamoudi and Bakrshoom 2021). *Vitex agnus-castus* is one of the most significant plants mentioned in reports for its therapeutic importance in gynecological diseases (Newall et al. 1996). It has traditionally been used to treat menstrual irregularities, and in particular, to help establish a normal menstrual cycle as well as to improve fertility *V. agnus-castus* does not contain hormones but reveals hormonal activity by affecting the pituitary gland, in particular to produce LH hormone. An adaptogenic effect on the anterior pituitary gland and regulation of LH secretion has been suggested by V. *agnus-castus*. It stimulates ovulation and increases the production of progesterone from the corpus luteum, which ultimately leads to the regulation of the female sexual cycle (Gerhard et al. 1998). *V. agnus-castus* extract will especially change levels of sex hormones until the imbalance disappears (Russo and Galletti 1995). *V. agnus-castus* extract has also been shown in reproductive biology to induce systematic ovulation, stabilize reproductive hormones, and promote regular menstrual cycles *V. agnus-castus* extract has been reported to surge the LH levels while gradually overcome the secretion of FSH and prolactin (van Die et al. 2013). Therefore, this study was conducted to focus on the effect of hydroalcoholic extract of the fruit of this plant on the serum levels of AMH, sex hormones, and $P450_{arom}$, *AMH*, and *CTRP6* expression in ovarian tissue following PCOS induction in rats.

Materials and Methods

Experimental design

Thirty-two sexually adult female Sprague Dawley rats (220 ± 30 g), with regular reproductive cycles (following 14 days of monitoring estrous cycles) were selected and randomly divided into four equal groups (n = 8).

The animals were maintained in standard polypropylene cages under controlled conditions 12/12-hr light/dark period and constant temperature (22 ± 2°C), and free access to a standard pellet diet and water. This study was ethically approved by the University of Shiraz (No. 98GCB1M1287)

Group1. Control group (C) (were not treated, and had free access to a standard pellet diet and water) received 0.2 ml of water by gavage daily.

Group 2. PCOS group (received estradiol valerate 4 mg/rat by a single intramuscular injection to induce PCOS), and received 0.2 ml of water by gavage daily.

Groups 3 and 4. Treatment groups I and II (these two groups were treated with 150 and 200 mg/kg *V. agnus-castus* fruit hydroalcoholic extract, respectively, for 30 consecutive days by gavage after induction of PCOS).

All investigations were conducted under the “Guiding Principles for the Care and Use of Research Animals” approved by the University.

Preparation of hydroalcoholic extract of *V. agnus-castus*

Fresh *V. agnus-castus* fruit was collected from farms in Fars province. The plants have been confirmed by a botanist from the Department of Biology, Shiraz University. The cleaned dried fruit of the plant was ground and the obtained powder was placed in 70% alcohol for 72 hours. After filtering, the extract was concentrated by a rotary machine under reduced pressure, and the residual alcohol was removed by placing the extract in an autoclave.

Monitoring the reproductive cycle

The reproductive cycle of the animals was monitored by vaginal smear for 14 days prior to selection for the experiments, after induction of PCOS to confirm induction of PCOS, and in the last eight days of the experiment (Figure 1). The stage of the estrous cycle was determined based on the ratio of the three types of cells which including: cornified, leukocytes and epithelial cells (Caligioni 2009).

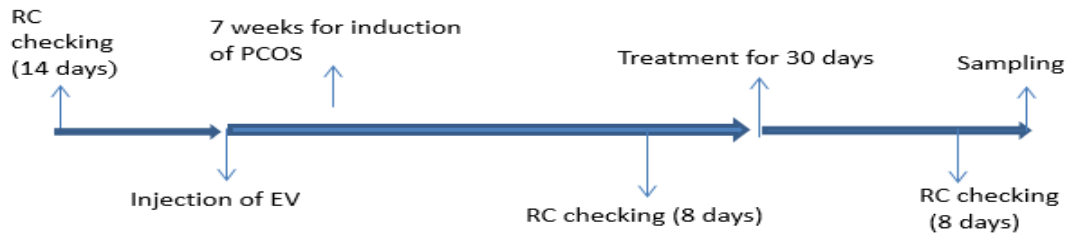

Figure 1. Schematic diagram of experimental design which includes Reproductive Cycle (RC) checking, estradiol valerate (SV) injection, treatment, and time of blood and tissue sampling

Induction of PCOS in animals

PCOS was induced in three groups (PCOS and two treatment groups) of rats with regular estrous cycles, seven weeks after a single intramuscular injection of 4 mg/rat of estradiol valerate dissolved in 0.2 ml of sesame oil (Mehraban et al. 2020). Estradiol valerate is a long-acting estrogen that disrupts the regular estrus cycle. Administration of this hormone causes hypothalamic-pituitary dysregulation of GnRH, resulting in abnormal release and storage of LH which is considered a crucial pathogenic factor in the development of PCOS (Shi and Vine 2012).

Blood sampling and ovary collection

The rats were anesthetized on the last day of the experiment, with carbon dioxide gas in a chamber for 3–5 min, euthanized by whole blood collection via heart puncture, and the ovaries in all groups were quickly removed. Serum and left ovaries were stored at -70°C until analysis. Ovaries were used to assess $P450_{arom}$, *AMH*, and *CTRP6* gene expression. Serum levels of AMH, estrogen, progesterone, testosterone, and DHEA were then measured.

Hormonal assay

Hormones including AMH were evaluated by a rat-specific ELISA kit (ZellBio, Germany), and sex hormones estrogen, progesterone, testosterone, and DHEA were measured in serum by the ELISA kit (Monobind, USA) according to the manufacturer's instruction.

Evaluation of $P450_{arom}$, *AMH*, and *CTRP6* expression in the ovaries

Left ovaries from all animals were used to evaluate $P450_{arom}$, *AMH*, and *CTRP6* expression. Total RNA was extracted by Total RNA Mini Kit (FAVORGEN Biotech, Taiwan) and quantitatively measured by a spectrophotometer (Thermo Scientific Nanodrop 1000, USA). To determine the concentration of DNA, absorption measurements at 260 and 280 nm were performed. Forty nanograms of template RNA were used to synthesize

cDNA following the instructions in the Fermentase Kit (K1621) cDNA Synthesis Kit (Thermo Fisher Scientific, USA) under the following conditions: 25°C for 10 min followed by 47°C for 60 min and 85°C for 5 min. cDNA samples were stored at -20°C. Next, quantitative PCR (qPCR) using RealQ Plus 2x Master Mix Green high ROXTM (AMPLIQON, Denmark) was performed on an Applied Biosystems (Waltham, Massachusetts, USA). Thermal cycles of qPCR reactions were as follows: 10 min of pre-denaturation at 95°C followed by 40 cycles for 15 sec at 95°C and 1 min at 60°C. Primer sequences are shown in Table 1. Primers for *P450$_{arom}$*, *AMH*, and *CTRP6* were designed from Primer3 (version 0.4.0) and synthesized by Metabion (Metabion, Germany). Also, the *β-actin* gene was used as an endogenous reference. All samples were assayed in triplicate. The comparative Ct ($2^{-\Delta\Delta Ct}$) method was used to calculate the expression levels of the genes

Statistical analysis

All data are presented as Mean ± standard error of the mean (Mean ± SEM). The software of SPSS version 22.0 for Windows was used for the statistical analysis of data. A one-way analysis of variance (ANOVA) was followed by a post hoc Tukey's multiple comparisons test, which was used to compare mean values among the groups. The significance level was set at <0.05.

Table 1. Primer sequences used for qPCR.

Genes	Primer Sequences	Sizes (Bp)
β-actin (ACTB)	Forward: **5'**- *CACCATTGGCAATGAGCGGTTC* -3' Reverse: **5'**- *AGGTCTTTGCGGATGTCCACGT* -3'	244
Cyp19 (p450$_{arom}$)	Forward: 5'- *TCCACACTGTTGTTGGTGACAG* -3' Reverse: *5'- AGCCGTCAATCACGTCATCC* -3'	150
CTRP6	Forward: 5'- *CGTTCGGGGTCTGTGAGTTG*-3' Reverse: 5'- *GTCCCCTTTGTCACCTTTCAGG* -3'	294
AMH	Forward: 5'- *ACCAAGCAAAGAAGGCTGTCC* -3' Reverse: 5'- *GAACCAAGCGAGTGAGGGTC* -3'	85

Results

Monitoring of the reproductive cycle

PCOS induced by estradiol valerate disrupted the estrous cycle in animals. Following the induction of PCOS, in most animals, the reproductive cycle was stopped in the estrus or diestrus stage, which is the criterion for entering animals into the study. Treatment with the extract caused resumption of an irregular estrous cycle (Table 2)**.**

Serum levels of AMH, Estradiol, Progesterone, Testosterone, and DHEA

Anti-Mullerian hormone levels in the PCOS group were significantly higher than in the control and both treatment groups (p=0.028, p=0.021, and p=002, respectively).

No significant difference in AMH levels was observed between the control and experimental groups (Figure 2).

Estradiol level in the PCOS group was significantly higher than the control group (p=0.028). The level of this hormone in the treatment group II declined compared with the PCOS group (p=0.028). There was no significant difference in estradiol levels between the control and treatment group II, however, the level of this hormone in treatment group I was significantly higher than the control group (Figure 2). There was no marked difference in estradiol levels between treatment group I and PCOS group (p=1.00).

There was no significant difference in progesterone levels between the PCOS, treatments, and control groups. Progesterone level in treatment group II

was significantly higher than the control group (p=0.031) (Figure 2).

Testosterone levels of the PCOS group were significantly higher than the control and treatment groups (p=0.028). There was no significant difference in the level of this hormone between both treatments and the control groups. DHEA levels did not change significantly among the groups (Figure 2).

Table 2. Duration of reproductive cycles (day) in study groups during experimental period (mean± SEM)

	control	PCOS	Treatment 1	Treatment 2
Beginning	4.8±0.19	4.7±0.35	4.6±0.70	4.6±0.34
	A	a	a	a
After Injection of estradiol valerate	4.6±0.14	--	-	--
	A	b	b	b
After treatment	4.4±0.18	--	4.7±0.66	4.8±0.44
	A	b	a	a

Different alphabets indicate a statistically significant difference between groups (p<0.05)

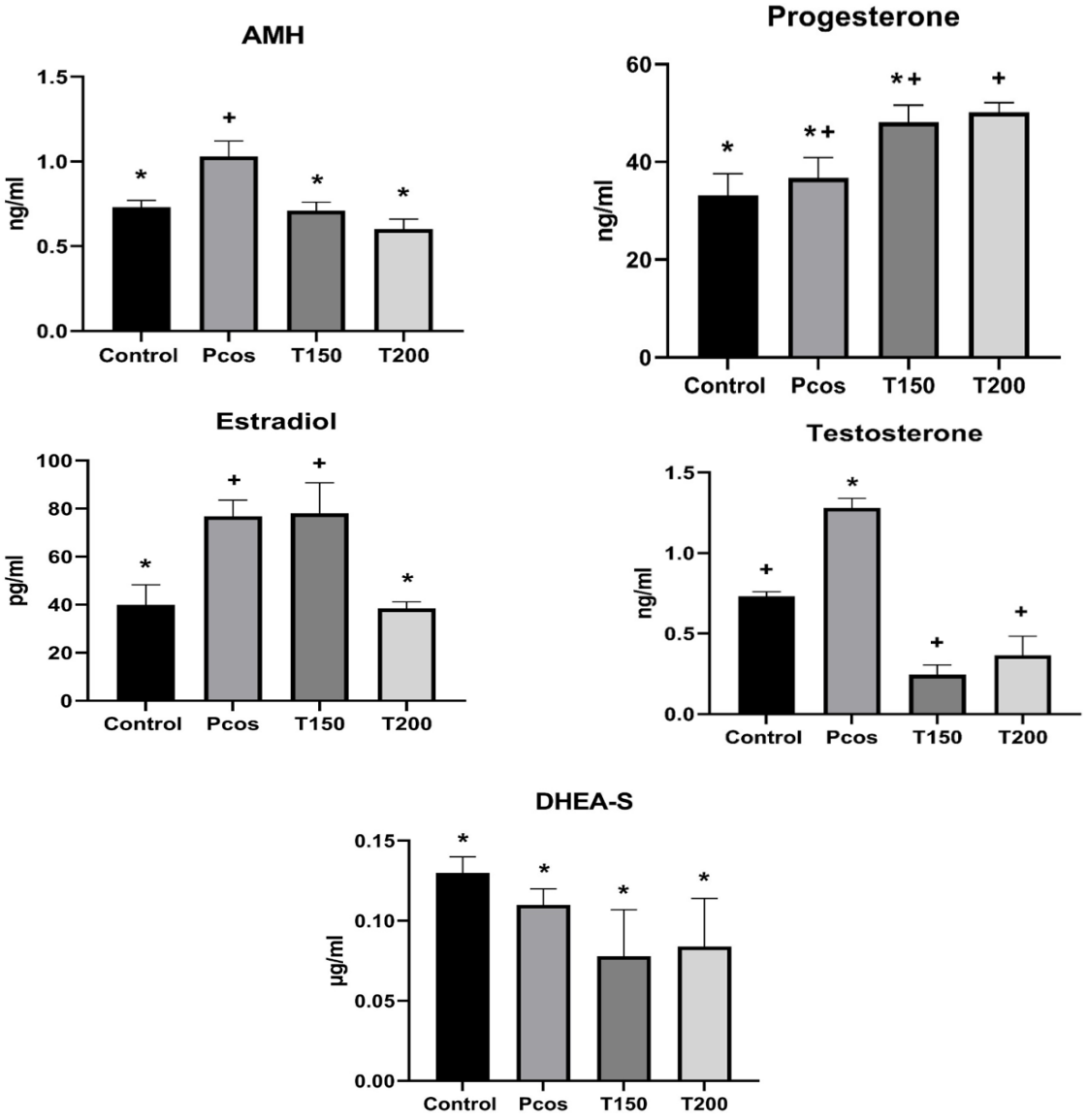

Figure 2. Comparison of serum levels of evaluated hormones in different groups. Data are presented as mean ± SEM, different symbols (*, +) indicate significant differences with other evaluated groups (p<0.05). Control (healthy animal without any treatment), PCOS(induced PCOS without treatment), T150 &T200 (animals with induced PCOS and treated with 150 or 200 mg of extract respectively)

Expression of *P450$_{arom}$*, *AMH*, and *CTRP6* gene in ovarian tissue

The mRNA expression level of *P450$_{arom}$* was significantly down-regulated in the PCOS group compared to the control (Figure 3). Treatment group II (treated with *V. agnus-castus* 200 mg/kg) showed significantly higher levels of *P450$_{arom}$* expression compared to the PCOS group and had no significant difference compared to the control group (p=0.253). The expression of this gene in treatment group I was not statistically significantly different from the PCOS group (p=0.999), but was significantly lower than the control group.

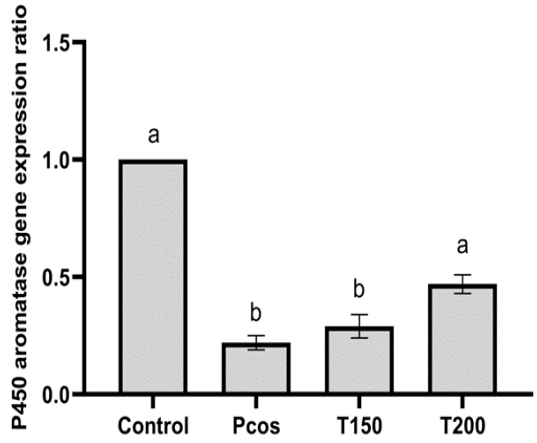

Figure 3. Quantitative analysis of the expression of P450arom in the ovarian tissue by real-time RT-PCR and effect of *V. agnus-castus* treatment on P450arom mRNA expression rate in different groups. Data are presented as mean ± SEM and different symbols indicate significant differences between groups (p<0.05). Control (healthy animal without any treatment), PCOS(induced PCOS without treatment), T150 &T200 (animals with induced PCOS and treated with 150 or 200 mg of extract respectively)

Based on Figure 4, *CTRP6* gene expression rate increased in the PCOS group compared to the other groups (p=0.0001). On the other hand, the expression of *CTRP6* gene significantly decreased in the treatment I and II groups compared to the PCOS group (p=0.0001). Expression of *AMH* also increased in the PCOS group compared to other groups, while treatment with *V. agnus-castus* extract significantly improved the condition in the treatment I and II groups (Figure 5) (p=0.003).

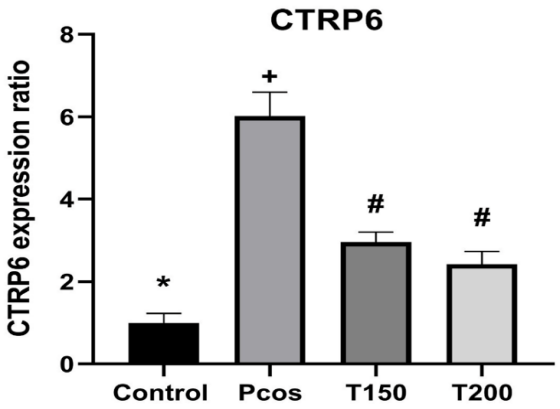

Figure 4. Quantitative analysis of the expression of CTRP6 in the ovarian tissue by real-time RT-PCR and effect of V.agnus-castus treatment on CTRP6 mRNA expression rate in different groups. Data are presented as mean ± SEM and different symbols indicate significant differences with other evaluated groups (p<0.05). Control (healthy animal without any treatment), PCOS(induced PCOS without treatment), T150 &T200 (animals with induced PCOS and treated with 150 or 200 mg of extract respectively)

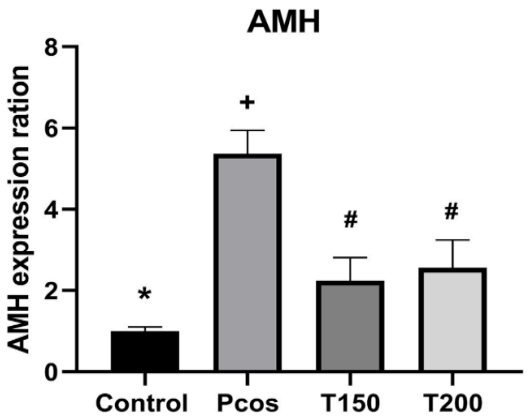

Figure 5. Quantitative analysis of the expression of AMH in the ovarian tissue by real-time RT-PCR and effect of *V.agnus-castus* treatment on AMH mRNA expression rate in different groups. Data are presented as mean ± SEM and different symbols indicate significant differences with other evaluated groups (p<0.05). Control (healthy animal without any treatment), PCOS (induced PCOS without treatment), T150 &T200 (animals with induced PCOS and treated with 150 or 200 mg of extract respectively)

Discussion

For centuries, *Vitex agnus-castus* has been considered an adaptogen to improve hormone imbalances. Because this herb extract also acts in other parts of the endocrine system to stimulate and stabilize the function of the pituitary gland, it is one of the most common herbs used to treat PCOS (Khanage et al. 2019). In the current study, the effect of *V. agnus-castus* fruit hydroalcoholic extract on PCOS status in a rat model was investigated. Following induction of PCOS by estradiol valerate, AMH, estradiol, and testosterone hormone levels were significantly increased. There was no significant difference in the DHEA level among the groups. Intramuscular injection of 4 mg estradiol valerate to induce PCOS in rats has been reported to significantly increase estradiol and testosterone levels (Mehraban, Jelodar et al. 2020). We did not observe a significant difference among the groups in DHEA levels, which is similar to results that have already been reported (Mehraban et al. 2020).

In this study, consumption of *V. agnus-castus* extract in rats with PCOS significantly decreased serum levels of estradiol, testosterone, and anti-Mullerian hormone, while having no significant effect on DHEA concentrations. Similar results have been reported following the administration of *V. agnus-castus* extract on estradiol, testosterone, and DHEA in animals with PCOS induced by letrozole (Jelodar and Askari 2012).

There was no significant difference in estradiol or AMH levels between the control and treatment group II. *V. agnus-castus* balances sex hormones with its physiological-pharmacological effects (Liu et al. 2004). Testosterone in the treatment groups was significantly lower than the PCOS group ($p<0.05$). This decrease may be due to the conversion of higher levels of androgens to estradiol through up-regulation of aromatase activity or down-regulation of AMH since the level of AMH is associated with testosterone concentration (Carlsen et al. 2009). Moreover, according to the available data, *V. agnus-castus* fruit extract contains some phytoestrogen compounds (Webster et al. 2006) and long-term use of phytoestrogens can also reduce testosterone through negative feedback on LH that may lead to a reduction of estradiol (Malaivijitnond et al. 2004). Treatment with 150 mg/kg *V. agnus-castus* extract significantly reduced the level of testosterone and AMH but had no significant effect on the concentrations of progesterone or estradiol. There was no significant difference in AMH levels between the control and treatment group I. *V. agnus-castus* extract did not have a significant effect on the concentration of DHEA. Since a significant percentage of DHEA is produced by the adrenal gland, and, *V. agnus-castus* mainly affects pituitary gonadotropins, no marked change in the level of this hormone is expected.

In PCOS, serum levels of the AMH are significantly increased. Recent studies have implicated both intra-ovarian and central activities of AMH hormone in the mechanism of anovulation and the etiology of PCOS (Franks and Hardy 2020). By growing follicles in the ovaries, *AMH* is expressed (Moolhuijsen and Visser 2020). It decreases the sensitivity of growing follicles to FSH and inhibits aromatase in granulosa cells in rodents, and as a result, inhibits follicle growth (Pellatt et al. 2010). In both rodents and humans, *AMHR2* is expressed in hypothalamic GnRH neurons. These neurons are responsive to AMH because the excitability and release of GnRH in rat's neuronal explants were stimulated by AMH (Cimino et al. 2016). Recently, the determination of serum AMH levels has been suggested as a valid indicator of ovarian function to evaluate female infertility and diagnose PCOS and possibly targeted infertility treatment (Neagu and Cristescu 2012). In the current study, the serum level of AMH was

increased significantly in the PCOS group. Treatment with the *V. agnus-castus* extract significantly reduced the level of AMH in treatment groups. There was no significant difference in the AMH levels between the control and treatment groups.

A deficiency in the acting of aromatase is one reasonable intra-ovarian disorder in steroidogenesis thought to cause PCOS (Rajan and Balaji 2017). Several investigations have reported low $P450_{arom}$ mRNA levels, a reduction in ovarian aromatase activity, and estrogen production in various sizes of PCOS follicles (Yu et al. 2013). Mice with a targeted disturbance of the *CYP19* gene have cystic follicles (Söderlund et al. 2005). A reduction in the action of this enzyme could result in rising ovarian androgen production and development of PCOS (Reddy et al. 2016). Our results showed a significant reduction in the mRNA levels of the $P450_{arom}$ in the PCOS group compared with the control group. Treatment with 200 mg/kg *V. agnus-castus* extract was also able to significantly increase the $P450_{arom}$ expression level in treatment group II compared to the PCOS group with no significant difference from the control group. Treatment with 150 mg/kg of *V. agnus-castus* extract did not have a marked effect on the $P450_{arom}$ expression level in treatment group I. Increased expression of *CTRP6* gene following induction of PCOS was observed in this study. Similar results have been reported in the animal model of PCOS, while treatment with vitamin D was reported to attenuate the expression of this gene (Hakimpour et al. 2022). Increased serum level of CTRP6 was also reported in women patients (Sadeghi et al. 2020) and it was proposed to be related to impaired glucose metabolism, as a major cause of PCOS. Treatments with *V. agnus-castus* extract have improved the expression of this gene in the ovaries possibly by improving endocrine or metabolic status.

Our results highlighted the positive effects of utilizing *V. agnus-castus* for the treatment of PCOS in an experimental model through endocrine and gene expression assessment. Although rats have been used and recommended as a model of PCOS for decades, it may not be a suitable and accurate model of this disease in humans.

In conclusion, administration of the *V. agnus-castus* fruit extract to the PCOS animals improved the endocrine profile and *AMH*, *CTRP6,* and $P450_{arom}$ gene expressions in the ovaries. The results highlighted the positive effects of utilizing *V. agnus-castus* for the treatment of PCOS in an experimental model.

Acknowledgment

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Conflicts of interest

The authors declare there is no conflict of interest to be mentioned

Funding

This research was financially supported by the Vice-Chancellor of Research of the University as a Ph.D. thesis.

Ethical Considerations

All investigations were conducted based on the “Guiding Principles for the Care and Use of Research Animals” approved by Shiraz University (98GCB1M1287) Code of Ethics.

Authors' Contributions

G.J designed, supervised and directed the project and edit the manuscript. M. A & A.S worked out almost all of the technical details, S.H participated in molecular part of this study.

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