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Effect of *Vitex agnus−castus* plant extract on polycystic ovary syndrome complications in experimental rat model

Amal H. Hamza[1,2✉], Widad M. AlBishri[1], Mona H. Alfaris[3]

[1]*Biochemistry Department, Faculty of Science, Jeddah University, Saudi Arabia*

[2]*Biochemistry and Nutrition Department, Faculty of Women, Ain− Shams University, Cairo, Egypt*

[3]*Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia*

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ABSTRACT

Objective: To investigate ameliorative effects of *Vitex agnus−castus* (VAC) and VAC containing pharmaceutical supplement (VPS) against polycystic ovary syndrome (PCOS).

Methods: PCOS in the rats was induced by daily administration of letrozole at 1 mg/kg body weight concentration for 21 d. PCOS rats were then treated daily either with metformin, VAC plant extract or VPS at 70, 8 or 8 mg/kg body weight concentration for 15 d. Rats that received none of these treatments were considered as control. Blood and ovaries were collected from all the rats. Serum glucose, cholesterol, triglyceride and high density lipoprotein-cholesterol were measured spectrophotometrically. Serum insulin, estrogen, progesterone, testosterone, luteinizing hormone, follicle-stimulation hormone, catalase, superoxide dismutase, malondialdehide and reduced glutathione were measured using enzyme-linked immunosorbent assay.

Results: Rats treated with letrozole demonstrated a significant increase in serum testosterone, estrogen, cholesterol, luteinizing hormone, triglycerides, glucose, insulin, and malondialdehide levels, and a significant decline in progesterone, follicle-stimulating hormone, high density lipoprotein-cholesterol, catalase and reduced glutathione levels compared to control. Contrarily, no significant change in superoxide dismutase was noted in response to letrozole treatment. Rats treated with metformin, VAC or VPS showed a remarkable reversal in the levels of parameters affected by letrozole treatment.

Conclusions: Data indicate that VAC and VPS exert potential ameliorative effects against PCOS through the modulation of hormonal and lipid profile as well as oxidative stress. Moreover, the favorable effects of these compounds are comparable to that of metformin.

1. Introduction

The most common endocrine metabolic dysfunction affecting 5%-20% women of reproductive age is polycystic ovary syndrome (PCOS). In accordance with the Rotterdam Workshop Group, 2003 definition of the diagnosis of PCOS in women was established with no or irregular periods, hyperandrogenism or the presence of polycystic ovaries[1]. Hormonal dysfunction, increased insulin level, stress, contraceptive pills and increased stimulation of adrenals are considered to be major risk factors in the development of PCOS[2]. The symptoms of PCOS may include hirsutism

✉First and corresponding author: Amal H. Hamza, Biochemistry Department, Faculty of Science, Jeddah University, King Fahd Road, Al Faisalia, Jeddah, Saudi Arabia; Biochemistry and Nutrition Department, Faculty of Women, Ain- Shams University, Cairo, Egypt.
Tel: +966501356650
E-mail: ahamza@kau.edu.sa; Amal_hamza@hotmail.com

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which is estimated to be present in about 70% of cases, menstrual irregularity, behavioral disorder, and subfertility[3]. Besides, PCOS is associated with impaired glucose tolerance, cardiovascular disease, anxiety, depression, metabolic syndrome, type 2 diabetes, obesity and infertility[4]. Additionally, reproductive dysfunction in PCOS is reported to induce endometrial hyperplasia, endometrial cancer, increased secretion of luteinizing hormone (LH), increased lipid profile, endothelial dysfunction and adrenal hyperandrogenism[1]. Several chemical agents have been used to induce PCOS in experimental animals such as dihydroepiandrosterone, estradiol valerate and letrozole[1]. Letrozole is a non-steroidal aromatase inhibitor, which prevents conversion of testosterone and androstenedione to estradiol and estrone. Letrozole induces PCOS by causing hyperandrogenism, hormonal dysfunction increased androgen synthesis, insulin resistance, and dyslipidemia[5].

The *Vitex agnus-castus* (VAC) is an herbal plant with multiple health benefits which are attributed to its various components including glycosides, flavonoids, progestins, alkaloids, volatile oil and essential fatty acids. VAC has been used in the treatment of hyperprolactinemia, infertility, cyclic mastalgia, abnormal menstrual cycles, bacterial and fungal infections[6–8]. VAC exerts its beneficial effects by stimulating LH release, ovulation and inhibiting follicle-stimulation hormone (FSH) release. VAC has also been shown to inhibit prolactin production by involving dopamine receptors. VAC is reported to abrogate corpus luteum defects, irregular menstrual cycles caused by hyperprolactinemia, breast pain, amenorrhea and premenstrual syndrome[9].

Different therapeutic methods have been recommended for PCOS treatment, including lifestyle changes, exercise, surgery, and medications. The medical treatment suggested for PCOS varies based on an individual's symptoms and may include metformin, clomiphene citrate, periodic progesterone, anti-androgens (spironolactone), and gonadotropin[10]. Metformin, an oral biguanide insulin-sensitizing drug, is the most widely used treatment for type 2 diabetes mellitus and PCOS worldwide. Metformin is also associated with increased menstrual cycle, improved ovulation, and reduction in circulating androgen levels[11].

Though VAC has been shown to alleviate the symptoms of PCOS, the underlying mechanism is not completely understood. Accordingly, here we investigated the mechanism through which VAC and VAC containing pharmaceutical supplement (VPS)[12,13] exert ameliorative effects against PCOS. Further, we compared the effects of these compounds with those of prescription drug, metformin. In the present study, we measured the effects of VAC, VPS and metformin on the levels of testosterone, progesterone, estrogen, LH, FSH, lipid profile, insulin, glucose and oxidative stress parameters in letrozone induced PCOS rat model. Moreover, we also examined the histological changes in the ovaries in response to letrozole and VAC treatment.

2. Materials and methods

Letrozole and metformin were purchased from Al-Nahdi Pharmacy, Jeddah, Saudi Arabia. Glucose, cholesterol, triglyceride and high density lipoprotein (HDL)-cholesterol kits were purchased from HUMAN, Germany. Insulin, estrogen, progesterone, testosterone, LH, FSH, catalase, superoxide dismutase (SOD), malondialdehide (MDA) and reduced glutathione (GSH) kits were purchased from NOVA., Beijing, China. VPS was purchased from GNC, Jeddah, Saudi Arabia.

2.1. Animals

Forty adult female Wistar Albino rats weighing 180-200 g were procured from the Animal House Colony of King Fahad Medical Research Center, Jeddah, Saudi Arabia. Rats were allowed to acclimatize to animal house conditions for two weeks. Rats were caged in standard polypropylene cages, maintained in controlled environment of 25 ℃ temperature and a 12 h light/dark cycle with free access to food and water. Present study was approved by the Ethical Committee of King Abdulaziz University and King Fahad Medical Research Center, Jeddah, Saudi Arabia with the protocol No. 171060175, dated December 2016.

2.2. Preparation of VAC plant extract

The VAC plant was obtained from Almadenah City, Saudi Arabia. Stems and leaves of the plant were rinsed with distilled water, dried and ground. The ground powder weighing 500 g was dissolved in 600 mL of ethanol and extracted in a Soxhlet apparatus. Ethanol was evaporated with a rotary machine and the residual was treated to form a powder. The resultant extract was then dissolved in saline before being orally administrated to animals. VPS was a commercially available VAC plant formulation in the form of tablets marketed by the name Agnucaston (Bionorica AG, Neumarkt, Germany). The use of commercially available VAC pharmaceutical supplements against PCOS was described previously[12,13].

2.3. Experimental design

Rats were divided into five groups with each containing 8 animals and designated as control, PCOS, PCOS+metformin, PCOS+VAC, and PCOS+VPS groups. Rats in PCOS group were treated daily with letrozone (dissolved in 0.5% carboxy methyl cellulose) at 1 mg/kg body weight concentration for 21 d to induce PCOS. Rats in PCOS+metformin group were treated with letrozone for 21 d followed by treatment with metformin at 70 mg/kg body weight concentration daily for 15 d[14]. Rats in PCOS+VAC group were treated with letrozone for 21 d followed by treatment with VAC plant extract at 8 mg/kg body weight concentration daily for 15 d. Rats

in PCOS+VPS group were treated with letrozone for 21 d followed by treatment with VPS at 8 mg/kg body weight concentration daily for 15 d. Control rats received 0.5% carboxy methyl cellulose orally as vehicle control for 21 d. At the end of the treatment regimen, rats were fasted overnight (12-14 h) and anaesthetized with diethyl ether and sacrificed by cervical dislocation. Blood samples were collected and serum was separated and used for biochemical analysis. Ovaries were dissected out directly from the lumbar dorsal wall beneath the inferior pole of the kidneys.

2.4. Biochemical analysis

Serum glucose, cholesterol, triglyceride and HDL-cholesterol were measured using commercially available kits following the manufacturer's instructions (HUMAN Germany Co., Ltd). Serum insulin, estrogen, progesterone, testosterone, LH, FSH, catalase, SOD, MDA and GSH were measured using enzyme-linked immuno sorbent assay based kits in accordance with the manufacturer's instructions (NOVA. Beijing, China).

2.5. Histological staining of ovary tissues

Histological staining of sections of ovaries was carried out according to the method described by Yoo and Lee[15] with minor modification. Briefly, immediately after dissection, ovaries were fixed in 10% formalin. Tissues were serially dehydrated in graded ethanol and xylene. Specimens were embedded in paraffin block and sections of 4-5 μm thick were cut and stained with hematoxylin and eosin stain and visualized under light microscope.

2.6. Statistical analysis

Data were analyzed by SPSS. Statistical differences between the groups were determined by one-way analysis of variance. Results were expressed as mean ± standard deviation (mean ± SD) (n=8) of 3 independent experiments. A P-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Reproductive hormone levels

The data on the serum levels of reproductive hormones in control and different treatment groups are presented in Figure 1. Data revealed a significant increase in testosterone and estrogen levels accompanied by a significant decrease in progesterone levels in rats with PCOS compared to healthy control group (P<0.05). PCOS rats treated with metformin showed a significant decrease in testosterone, and estrogen levels and a significant increase in progesterone compared to PCOS group. Consistently, a significant regression in testosterone, and estrogen levels accompanied by a significant increase in progesterone levels were noticed in PCOS rats treated with either VAC or VPS compared to PCOS rats.

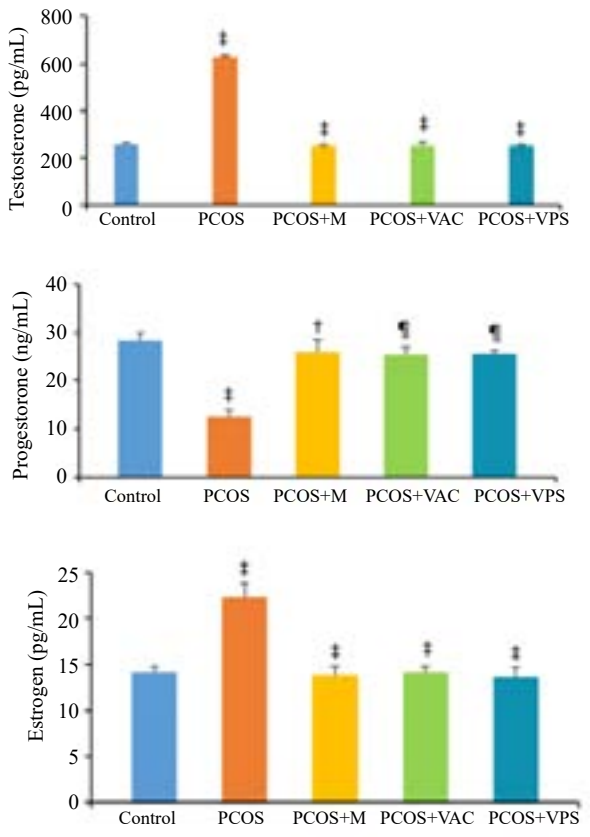

Figure 1. Reproductive hormone levels in control and different treatment groups of rats.

PCOS group was compared to control group, while PCOS+M, PCOS+VAC or PCOS+VPS groups were compared to PCOS group. †P<0.05, ¶P<0.01, ‡P<0.001. PCOS: polycystic ovary syndrome, M: metformin; PCOS+VAC: PCOS+*Vitex agnus-castus*, PCOS+VPS: PCOS+*Vitex agnus-castus* pharmaceutical supplement. The data represent mean± SD of 3 independent experiments (n=8).

3.2. LH and FSH levels

The data on the serum levels of LH and FSH in control and different treatment groups were presented in Figure 2. A significant increase in LH accombined by significant decrease in FSH levels was observed in rats with PCOS compared to healthy control group (P<0.05). PCOS rats treated with metformin as well as VAC or VPS showed a significant improvement in LH and FSH levels compared to PCOS group.

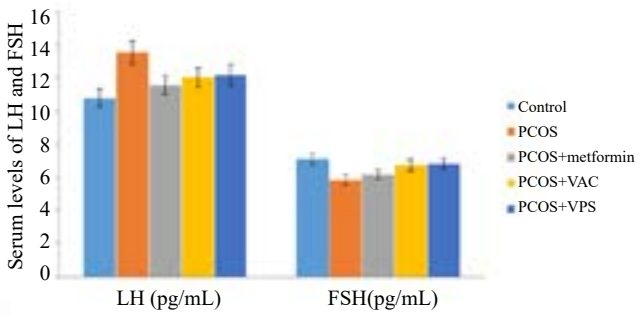

Figure 2. LH and FSH levels in control and different treatment groups of rats.

PCOS group was compared to control group, while PCOS+M, PCOS+VAC or PCOS+VPS groups were compared to PCOS group.‡P<0.001. LH: leutinizing hormone; FSH: follicle stimulating hormone; PCOS: polycystic ovary syndrome; PCOS+VAC: PCOS+V*itex agnus-castus*; PCOS+VPS: PCOS+*Vitex agnus-castus* pharmaceutical supplement. The data represent mean±SD of 3 independent experiments (n=8).

3.3. Lipid profile

The data on serum lipid profile of control and different treatment groups were provided in Table 1. A significant elevation in cholesterol and triglyceride levels accompanied by a significant decrease in HDL-cholesterol levels were observed in rats with PCOS as compared to a healthy control rats. On the other hand, metformin, VAC or VPS treated rats demonstrated a significant reduction in cholesterol and triglyceride level and a significant increase in HDL-cholesterol level as compared to PCOS rats.

Table 1. Lipid profile in control and various treatment groups of rats.

Groups	Total cholesterol (mg/mL)	HDL-cholesterol (mg/dL)	Triglycerides (mg/dL)
Control	215.6 ± 1.9	160.7 ± 1.0	80.1 ± 1.3
PCOS	240.1 ± 1.3‡	144.4 ± 1.2‡	120.2 ± 0.8‡
PCOS+metformin	220.2 ± 0.8‡	155.3 ± 1.7‡	90.1 ± 1.4‡
PCOS+VAC	225.1 ± 2.0‡	150.3 ± 1.1‡	100.2 ± 1.8‡
PCOS+VPS	230.3 ± 3.4‡	152.3 ± 1.0‡	95.2 ± 1.2‡

PCOS: polycystic ovary syndrome; PCOS+VAC: PCOS+*Vitex agnus-castus*; PCOS+VPS: PCOS+*Vitex agnus-castus* pharmaceutical supplement. The data represent mean ± SD of 3 independent experiments (n=8). PCOS group was compared to control while PCOS+metformin, PCOS+VAC or PCOS+VPS groups were compared to PCOS group. ‡P<0.001.

3.4. Glucose and insulin levels

The data on serum glucose and insulin levels in control and different treatment groups were presented in Figure 3. Compared to control, there was a marked increase in serum glucose and insulin levels in letrozone treated PCOS rats (P<0.05). The treatment of PCOS rats with metformin, VAC or VPS resulted in a significant regression in both glucose and insulin levels compared to letrozone treated PCOS rats (P<0.05).

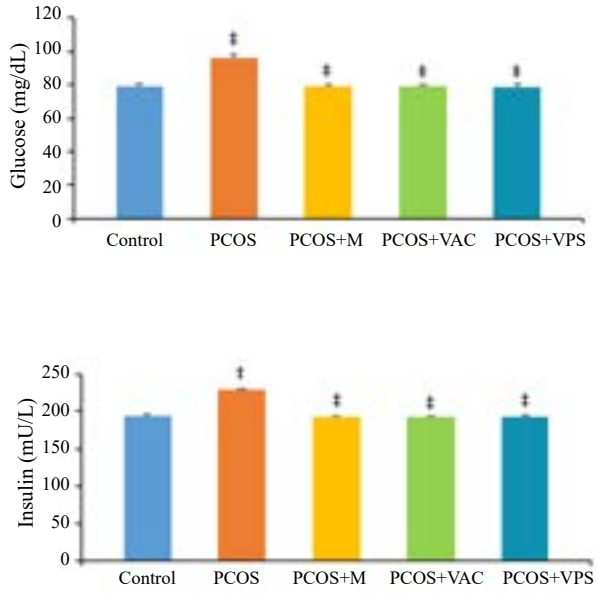

Figure 3. Glucose and insulin levels in control and different treatment groups of rats.

PCOS group was compared to control group, while PCOS+M, PCOS+VAC and PCOS+VPS groups were compared to PCOS group. ‡P<0.001. PCOS: polycystic ovary syndrome; M: metformin; PCOS+VAC: PCOS+*Vitex agnus-castus*, PCOS+VPS: PCOS+ *Vitex agnus-castus* pharmaceutical supplement. The data represent mean±SD of 3 independent experiments (n=8).

3.5. Oxidative stress markers

Effects of letrozole, metformin, VAC and VPS on serum oxidative stress markers were shown in Table 2. The MDA levels were significantly augmented while GSH and catalase levels were significantly downregulated in PCOS rats compared to control, whereas no significant effect was observed in SOD levels. The PCOS rats receiving VAC or VPS had significantly attenuated MDA levels. No significant change in MDA levels was noticed in metformin treated PCOS rats. Neither metformin, VAC or VPS treated PCOS rats had any effect on GSH levels. On the other hand, only metformin significantly increased SOD levels in PCOS rats. Catalase was significantly elevated in response to metformin, VAC or VPS treatment of PCOS rats.

Table 2. Oxidative stress markers in control and various treatment groups of rats.

Groups	MDA (ng/mL)	GSH (pg/mL)	SOD (pg/mL)	Catalase (pg/mL)
Control	3.2 ± 1.6	6.9 ± 1.2	7.3 ± 1.4	19.3 ± 0.7
PCOS	5.1 ± 1.2†	4.9 ± 1.3¶	5.6 ± 1.6	14.4 ± 1.0‡
PCOS+metformin	3.8 ± 1.7	6.0 ± 0.9	7.8 ± 1.6†	18.6 ± 0.6‡
PCOS+VAC	2.8 ± 1.6¶	5.0 ± 1.2	6.4 ± 2.1	19.2 ± 0.7‡
PCOS+VPS	2.9 ± 1.6‡	5.4 ± 1.5	6.2 ± 2.2	17.7 ± 1.0‡

PCOS: polycystic ovary syndrome; PCOS+VAC: PCOS+ *Vitex agnus-castus*; PCOS+VPS: PCOS+*Vitex agnus-castus* pharmaceutical supplement; MDA: malondialdehyde; GSH: reduced glutathione; SOD: superoxide dismutase. The data represent mean±SD of 3 independent experiments (n=8). PCOS group was compared to control group while PCOS+metformin, PCOS+VAC or PCOS+VPS groups were compared to PCOS group. $^{\dagger}P$<0.05, $^{\P}P$<0.01, $^{\ddagger}P$<0.001.

3.6. Histology of ovaries

Histological evaluation in the ovaries of control and different treatment groups were provided in Figure 4. In healthy control group normal histological structure of ovary was observed. On the other hand, In PCOS rats, absence of corpora lutea, fewer atretic follicles with fluid filled antrum and higher incidence of pyknotic granulosa cells were observed compared to normal histology of ovaries in control. In VAC or VPS treated rats, normal follicles, blood vessels, disappearance of cysts and healthy corpora lutea were noticed. Furthermore, increased corpora lutea and normal sized healthy follicles at different developmental stages with oocytes were observed.

4. Discussion

The most common hormonal disorder diagnosed in women of reproductive age is polycystic ovaries. PCOS is a hormonal and metabolic disorder that affects women, and leads to infertility and poor reproduction[16]. Cardiovascular disease and type 2 diabetes are the main complications associated PCOS in women[17]. In the present study, letrozole was used to induce PCOS in female Wistar rats. Previous studies suggest that letrozole-induced PCOS condition depicts human PCOS in many ways[18].

As evident in our results, marked increase in testosterone, estrogen and LH accompanied by decreased progesterone and FSH hormone levels compared to control confirms the development of hyperandrogenism in PCOS rats. In the present study, we found a significant increase in the levels of glucose and insulin in PCOS rats indicating the presence of insulin resistance. This is in congruity with the previous findings which reported induction of hyperglycemia and insulin resistance by letrozole[19]. The observed hyperglycemia and hyperinsulinemia may be due to a defect in insulin binding caused by decreased receptor binding or defect at the level of glucose transporters and the activities of other enzymes involved in glucose metabolism. One of the consequences of PCOS is also the imbalances in lipid profile and the development of dyslipidemia[20]. Our study exhibited similar results in lipid profile. PCOS-induced group showed notable increase in cholesterol, triglyceride and decrease in HDL levels. The differences in hormones levels and lipid profile are attributed to hyperandrogenemia[21]. The adverse effects of excess androgen may be manifested in several systems. Androgen

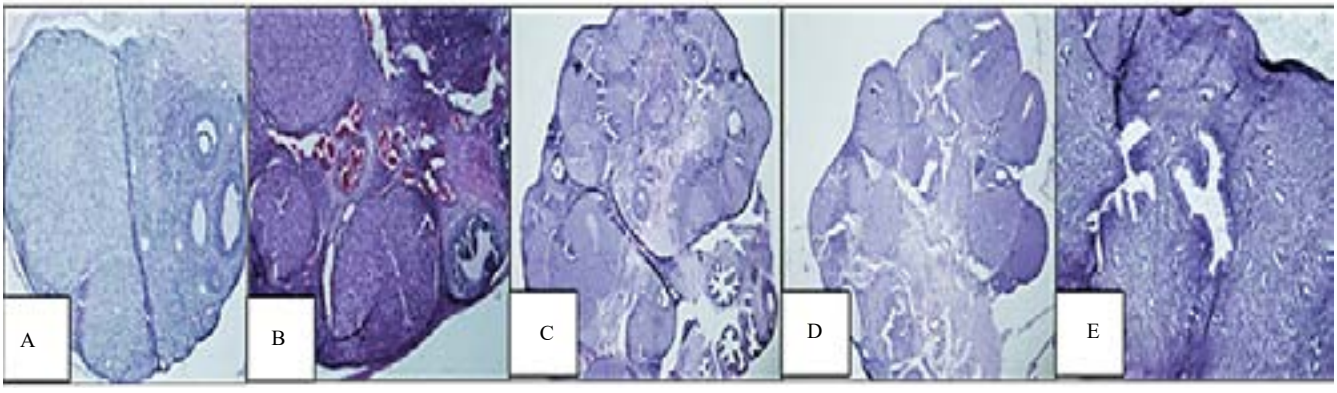

Figure 4. Hematoxylin and eosin stained sections of ovaries from control (A), PCOS (B), metformin treated (C), VAC treated (D) and VPS treated (E) rats. Normal histological structures could be seen in control rats, while absence of corpora lutea, few follicles and atretic follicles containing fluid filled antrum and higher incidence of pyknotic granulosa cells were seen in PCOS rats. Rats tretaed with metformin, VAC or VPS showed presence of corpora lutea, absence of cysts, normal sized healthy follicles and decreased fluid filled antrum and incidence of pyknotic granulosa cells. PCOS: polycystic ovary syndrome,VAC: *Vitex agnus-castus*, VPS: *Vitex agnus-castus* pharmaceutical supplement. (Images were presented at 40 × magnification).

receptors present on adipocytes and testosterone have an antilipolytic effects on abdominal subcutaneous preadipocytes, apparently through selective inhibition of catecholamine-induced lipolysis[21,22]. High testosterone levels presumably reflected accumulation of androgens possibly due to the blockade of conversion of androgen substrates into estrogens. The high serum LH concentrations could be due to the reduction of estrogen production in hypothalamus and pituitary due to the letrozole presumably enhanced LH secretion[23]. The PCOS is also associated with oxidative stress which leads to increased androgen production[24]. In PCOS, increased formation of lipid hydroperoxides and high levels of MDA could be due to the increased oxidation of biomolecules leading to extensive lipid peroxidation in proteins, membranes, and genes. The high levels of MDA are indicator of free-radical mediated damage to the tissue[25].

In this study, treatment with metformin significantly ameliorated hormones, lipid profile, glucose and insulin as well as oxidative stress. Metformin has been extensively used for PCOS treatment, especially in women with hyperglycemia and insulin resistance. Despite the described beneficial effects of metformin on ovarian physiology, the mechanisms of action of this drug in the ovary are still unclear. Metformin, antihyperglycemic drug, has been shown to improve hyperandrogenism and hyperinsulinemia, most likely through its positive effects on glucose utilization in insulin-sensitive tissues. In the current work, PCOS rats treated with metformin recorded an improvement in all parameters studied. These results may be due to the pharmacokinetics and pharmacodynamics of metformin which can be acted as steroidogenic agent. Our findings are consistent with previous studies[26,27].

Treatment with VAC either as alcohol extract or as pharmaceutical supplement significantly reversed the levels of hormones, lipid parameters, glucose, insulin and oxidative stress markers as compared to PCOS. It appears that VAC plant extract is more effective in the modulation of circulating androgen profile than VPS. It may be suggested that VAC's hypoandrogenic effect could be related to its active flavonoid compound. Presence of flavonoids in several plants has the ability to inhibit testosterone secretion by interfering the effect of insulin on growth factor-1 in ovarian stroma[28]. The hypolipidemic effects of VAC are well documented in previous studies[29,30]. For example, VAC effectively minimized the atherogenic lipid profile in rodents with hyperlipidemia and PCOS complications due to its flavonoid, diterpenoids, and iridoids content[30]. VAC extract also contains the complex mixture of total phenolic and flavonoid compounds that are antioxidants. Studies have indicated that the phytochemicals such as flavonoids, carotenoids and other phenolic compounds provide significant antioxidant activity and health benefit. This could account for the high antioxidant activity of VAC and thus was considered as naturally occurring potential antioxidant source[31]. The histopathological results strongly support the biochemical results obtained in this study. Treatment with metformin, VAC or VPS effectively reversed the PCOS induced damage to ovaries.

In conclusion, we demonstrated that VAC plant extract exerts multiple beneficial effects similar to metformin in treating PCOS condition and inducing ovulation. VAC effectively reversed the PCOS induced changes in hormones, lipid profile, oxidative stress and glycemic status. These effects may be ascribed to its several pharmacological properties including antihyperlipidemic, antioxidant and hypoglycemic effects which could be useful in managing PCOS condition. These beneficial impacts of VAC make it a promising agent for the treatment and prevention of PCOS.

Conflict of interest statement

All authors declare that they have no conflict of interest.

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