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Effect of *Glycyrrhiza glabra* Extract with and Without Vitamin E on Induced Polycystic Ovary (PCOS) in the Rats

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Abstract: Polycystic ovary syndrome (PCOS) is a diverse endocrine and metabolic disorder with a hereditary predisposition. Conventional hormonal and surgical therapies have given non satisfactory results. The purpose of the current work was to inspect the efficacy of *Glycyrrhiza glabra* (licorice) extract and vitamin E combinations on ovarian performance in induced polycystic ovary (PCOS). For this study, a well-prescribed rat model using daily Letrozole oral administration (1 mg/kg b.wt) over 21 days had been recruited after that, the rats received Licorice extract (300 mg/kg b.wt), vitamin E (400 mg/kg b.wt) or the combination of Licorice and vit E for 28 days. Serum samples were taken for hormonal evaluation of testosterone and beta subunit of LH and metabolic profile assessment of blood glucose level and lipid profile. In addition, the antioxidant status of ovaries and histopathological examination were conducted. The findings revealed that treatment with plant extract with and without vitamin E exerted beneficial effects in PCOS rats evidenced by improving the antioxidant status of ovaries, the metabolic status and hormonal profile of rats. However, the combination showed more prominent effects. Finally, it was concluded that the combination of licorice and vit. E can used as encouraging treatment for poly cystic ovaries syndrome as it enhances the antioxidant status and hormonal profile of rats.

Key words: Licorice · Vit. E · PCOS · Antioxidant

INTRODUCTION

Polycystic ovary syndrome (PCOS) is considered from the major reproductive problem affected women and animals. In women, PCOS hits 5-10 % of them in the reproductive age [1] and characterized by amenorrhea, anovulation, hirsutism, impaired menstruation and infertility [2]. Endale *et al.* [3] stated that, ovarian cyst is one of the most prevalent reasons of reproductive impairment and economic losses in the dairy sector. Although the pathophysiology of PCOS is still unknown, hyperandrogenemia is generally accepted to be the root motive of PCOS which intensified by excess androgen production from several tiny follicles [4]. Different clinical and metabolic complications are moreover related to PCOS such as diabetes, coronary artery disorder, hypertension, unremitting oligo-ovulation, anovulation and cancers of breast, ovaries and endometrium [5]. Several studies have stated that oxidative markers are markedly increased in PCOS and regarded as a contributing factor of PCOS pathophysiology [6].

The current care standard of PCOS includes everything from lifestyle changes to pharmaceutical and surgical procedures. Antiandrogens (Flutamide and Spironolactone), insulin-resistance medicines (Thiazolidinediones and Metformin) and estrogen-progestin compound (Oral contraceptives) are some of the pharmacological options, Such treatment is costly and can cause a variety of unpleasant side effects, including gastrointestinal issues, irregular menstruation, increased insulin resistance and weight gain as well as being ineffective in some cases [7]. Several studies are devoted to the study of complementary and alternative medicine, herbal medicines are promising in PCOS treatment [8]. Phytoestrogens act as estrogen agonists or antagonists can also be classified as selective estrogen receptor modulators have a potential effect on PCOS treatment [9].

Licorice (Glycyrrhiza spp.), the traditional medical herb extensively grown in the Southwest Asia and Mediterranean regoin. It comprises numerous pharmacological elements. Flavonoids, isoflavones, glycyrrhizin, glabridin, beta-Glycyhrritinic acid, chalcones

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and triterpenoid saponins are among these components [10]. Glabridin is a significant component of licorice and has numerous biological actions, including antibacterial, anti-osteoporosis, neuroprotective, estrogenic and antioxidant properties [11]. licorice increased aromatization of testosterone to 17 beta oestradiol that could be considered an adjuvant therapy for PCOS [8, 12].

Vitamin E (alpha tocopherol) is a fat-soluble vitamin with non enzymatic antioxidant properties and plays an important role in reproductive processes. It can reduce oxidative stress caused by antioxidation imbalance and free oxygen radicals by inhibiting the activities of phospholipase A and lipoxygenase, thereby stabilizing cell membranes and regulating the normal physiological functions of the reproductive system [13]. It exhibits chemoprotective effect against degenerative changes in ovarian tissue [14]. Vitamin E provides antioxidant and may also modulate the antiestrogenic effect of clomiphene citrate [15]. Based on the mentioned above, the present investigation was established to identify the efficacy of *Glycyrrhiza glabra* hydroalcoholic extract along with potential benefits of specific combinations with vitamin E for treatment of hyperandrogenism-induced PCOS.

MATERIALS AND METHODS

Preparation of Licorice Extract: *Glycyrrhiza glabra* (licorice) will be identified at the Herbarium of the Department of Botany, Faculty of Science, Cairo University. The dried roots and rhizomes were crushed and extracted with 70% v/v ethanol using Soxhlet's apparatus. The hydroalcoholic extract of *G. glabra* were pooled and concentrated under reduced pressure and evaporated in air to dry. The alcoholic extract was chilled in refrigerator until use [16].

Experimental Animals: The experimental protocol was approved by the Institutional Animal Care and Use Committee of Cairo University (CU-IACUC; VetCU11112018017). Virgin, cyclic, adult female Wistar rats (160–200 g) were used for this study. rats were obtained from Laboratory of Animal Colony, Helwan, Egypt and were housed in animal house of the Faculty of Veterinary Medicine, Cairo University, caged in standard polypropylene cages, sustained in controlled environment of (22 ± 3)°C temperature, (55 ± 5) % humidity and a 12 h light/ dark cycle and fed on standard diet, the food and water were allowed *ad libitum*. The rats were acclimatized for two weeks prior the starting of experiment.

Experimental Protocol: Forty female rats were randomly allocated into five groups of 8 each as following, group one was kept as the control group whereas the rats received daily oral dose (1ml) of the distilled water using a gavage. PCOS was induced, rats of groups 2 to 5 as guided by an established rat model outlined [17] whereas, rats received letrozole (LTZ) (Natco Pharma Limited Hyderabad) 1 mg/kg dissolved in 0.5% CMC daily for 21 days. After induction of PCOS, rats were given orally the different treatment for 28 days, Group 2 (PCOS) received only the vehicle; Groups (3-5) were treated with Licorice extract 300 mg/kg [18], vitamin E400 mg/kg [19] and licorice extract with vitamin E. From the sixth day of the study, daily vaginal smears of all rats were examined to check ovulation where, indiscriminate estrous cycle with prolonged diestrous phase indicated PCOS [20]. On 50th day of the study, rats were anesthetized with ketamine 91 mg/kg, i.p. and blood samples were collected into gel separator tubes to obtain serum samples, where they centrifuged at 3000 g at 4°C for 10 min and separated to estimate different biochemical parameters and hormonal profile assessment. Rats were then sacrificed, ovaries and uteruses excised, cleaned of fat and weighed and divided into duplicates; one set; stored at –80°C to be used for antioxidant assays. Other set were fixed in 10% neutral buffered formalin for histopathological examination. After weighing the body and ovaries of each animal at the day of scarification, the relative weight of ovary was calculated as the ratio of ovary (wet weight, mg) to body weight (g) as explained in Fig. (1).

Hormonal Profile: Competitive ELISA kits (Chemux Bioscience Inc, USA) were used for measuring total testosterone hormone level and rat lutropin subunit beta ELISA kit (EIAab, China) for estimating beta subunit chain of LH level following the instructions of the manufacturer [21]. Sensitivity = 0.05 ng/ml & less than 0.15 mlU/ml, intra-assay Precision C.V. (%) = 7.43 and <15 and, inter-assay Precision C.V. (%) = 5.23 and <15 for testosterone and rat lutropin subunit beta, respectively.

Biochemical Parameters: The collected serum samples were used for assessing glucose level and lipid profile (Cholesterol, triglycerides and high density lipoprotein (HDL) cholesterol) using commercial kits (spectrum, Egypt) [22, 23] while; very low density lipoprotein (VLDL) cholesterol concentration = Triglycerides/5 and low density lipoprotein (LDL) cholesterol concentration = Total cholesterol – (HDL + VLDL) according to Friedewald *et al.* [24].

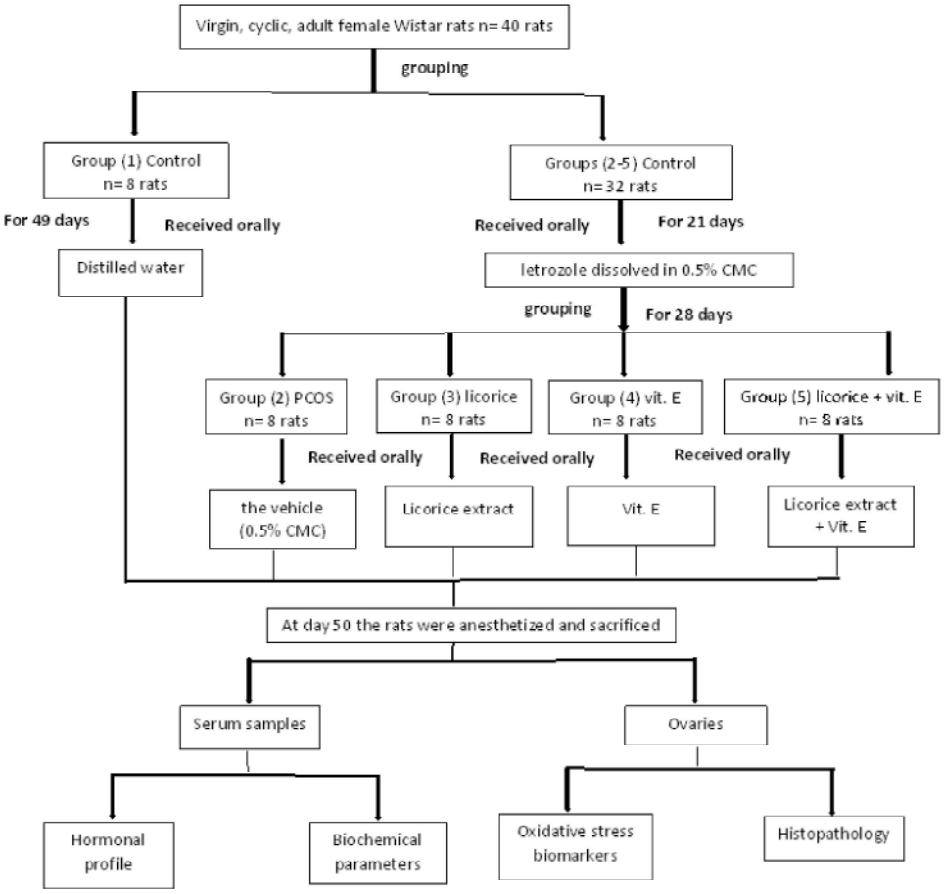

Fig. 1: Graphical design of experiment

Osidative Stress Biomarkers: Using glass homogenizer, ovarian tissues were homogenized in 10 ml cold buffers per gram tissue, their pH is 7.5, the buffers were 50 mM potassium phosphate + 1 mM EDTA for reduced glutathione (GSH), while 50 mM potassium phosphate for MDA, finally the homogenates were obtained, centrifuged at 15000 rpm for 15 min, the supernatant were collected to measure the activity of reduced glutathione (GSH) and MDA concentration according to protocols of Beutler *et al.* [25] and Ohkawa *et al.* [26].

Histopathological Examination: Ovaries from different groups were collected and fixed in 10% neutral-buffered formalin and processed to obtain 3-4 µm paraffin embedded sections. The sections were stained with hematoxylin and eosin (H&E) and the morphometric analysis of the ovaries were performed [27].

Statistical Analysis: Data for multiple variable comparisons was analyzed by one-way analysis of variance (ANOVA) test to analyze the significant differences ($P < 0.05$) between groups using SPSS version 24 package for Windows, results are expressed as the mean ± SD. Duncan's post hoc was used and least significant difference tests to check the inter group comparison. Figures were created using Graph Pad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

Hormonal Profile: Induction of PCOS significantly increased LH and testosterone levels compared with the control group. Following 28 days of treatment, licorice extract and vitamin E lowered ($p < 0.05$) LH and testosterone levels. The combination group displayed superior hormonal effect where it exhibited no significant difference when compared with control group (Fig. 2).

Biochemical Parameters: The results of lipid profile and glucose level (Table 1) showed that, total cholesterol, triglyceride, LDL cholesterol, VLDL cholesterol

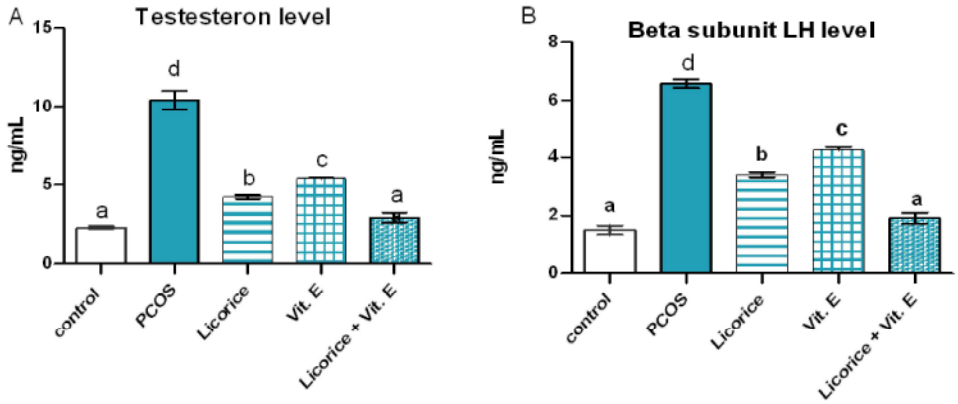

Fig. 2: Effect of *Glycyrrhiza glabra* (Licorice extract) and/or vitamin E on testosterone and beta subunit LH levels in letrozole induced PCOS rat model. Data presented as (Mean ± SD), different alphabets are statistically significant compared to control group at $P < 0.05$

Table 1: Effect of *Glycyrrhiza glabra* extract (licorice) and/ or Vitamin Eon lipid profile and glucose level in letrozole induced PCOS rat model

Parameters (mg/dL)	Control	PCOS	Licorice	Vit E	Licorice+ Vit E
Cholesterol	97.8± 4.2[a]	138.78± 1.6[c]	100.3± 5.94[a]	112.9± 2.64 [b]	99.6± 4.73[a]
Triglycerides	36.12± 4.1[a]	62.64± 3.41[c]	38.99± 3.69[a]	48.52± 0.86[b]	37.23± 3.11[a]
HDL Chol.	72.17± 6.1[a]	68.42± 5.1 [a]	74.11 ± 5.08[a]	78.23 ± 4.38[a]	72.44 ± 3.02[a]
LDL Chol.	18.66± 2.2[a]	57.82± 0.4[b]	19.01± 1.49[a]	24.07 ± 1.03[a]	19.66± 2.13[a]
VLDL Chol.	7.22± 0.4[a]	12.53± 0.8[c]	7.8± 0.74[a]	9.7 ± 0.17[b]	7.5± 0.62[a]
Glucose	92.06± 4.3[a]	148.4 ± 0.8[c]	94.13 ± 2.5[a]	114.4 ±2.9[b]	93.34± 1.2[a]

Data presented as Mean ± SD, different superscript statistically significant compared to control group (normal) at p<0.05 (n=5).

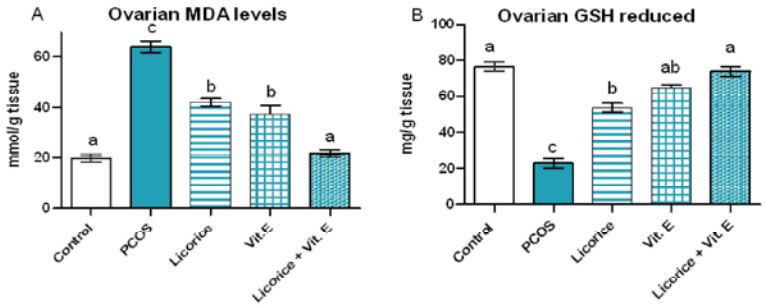

Fig. 3: Effect of *Glycyrrhiza glabra* (Licorice extract) and/or vitamin E on ovarian MDA level and reduced glutathione activity in letrozole induced PCOS rat model. Data presented as (Mean ± SD), different alphabets are statistically significant compared to control group at $P < 0.05$

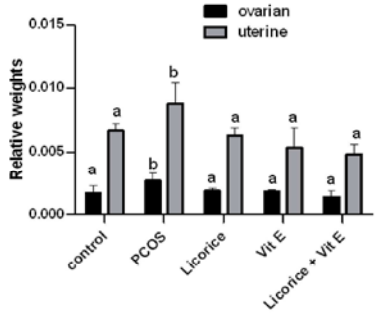

Fig. 4: Effect of *Glycyrrhiza glabra* (Licorice extract) and/or vitamin E on relative ovarian and uterine weights in letrozole induced PCOS rat model. (Mean ± SD), different alphabets are statistically significant compared to control group at $P < 0.05$ (n=5)

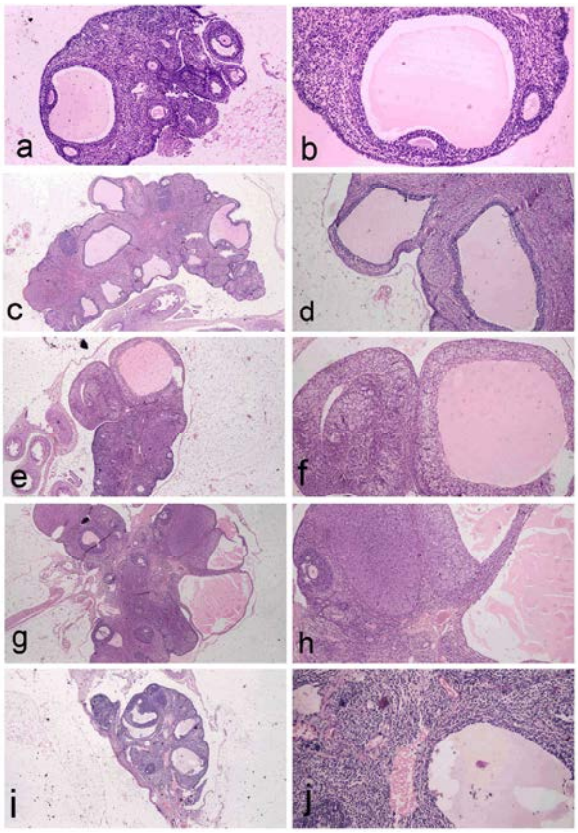

Fig. 5: Histopathological pictures of ovaries in different groups with H&E stain X40. The left picture with higher magnification power X200. (a-b) control group showing normal histological picture of ovary. (c-d) polycystic group showing multiple multiple ovarian cysts. (e-f) licorice treated group showing only one ovarian cyst. (g-h) Vit. E treated group showing two ovarian cysts. (i-j) licorice +Vit.E treated group showing reduction in the number of ovarian cysts

concentration and glucose level significantly elevated in PCOS group in comparison to the control group. Licorice extract and its combination with vitamin E groups exhibited no significant change in lipid profile compared to control rats, while vitamin E group showed great enhancement in lipid profile and glucose level than PCOS group.

Oxidative Stress Biomarkers: The data of oxidative stress markers in ovary presented in Figures 3 revealed that, induction of PCOS significantly augmented MDA level and down regulated reduced glutathione (GSH) activity in comparison with the control group. After 28 days of treatment, the ovarian MDA levels reduced ($p < 0.05$) and GSH activity significantly upregulated in licorice extract vitamin E groups. The combination group displayed better MDA level and GSH activity results where they didn't differ significantly when compared with control rats.

Relative Ovarian and Uterine Weights: The relative weights of both organs were increased in PCOS group. However, these figures reduced in all groups after treatment and showed no significant change compared to control (Figure 4).

Histopathological Examination: The control group showed normal ovarian tissues, with several follicles at varying stages of development and normal granulosa, theca and mesenchymal stromal cell layers, as well as various corpora lutea (Fig. 5a, b). The PCOS group showed several ovarian cysts as well as tiny follicles in the early stages of development, but no signs of corpora lutea. The cystic follicle's follicular walls were lined by a thin layer of flattened granulosa cells, the majority of which were necrotic and apoptotic. Theca interna and extrna proliferated markedly (Fig. 5c, d). The ovarian functions were recovered in the groups treated with licorice (Fig. 5e, f), vitamin E (Fig. 5g, h) and licorice combined with vitamin E (Fig. 5i, j), with a lowering in the count of ovarian cysts, restoration of granulosa cell thickness and presence of corpora lutea.

DISCUSSION

PCOS was induced in this study with the goal of determining the effects of *Glycyrrhiza glabra (licorice)* extract, vitamin E and their combination on testosterone, LH hormones levels, metabolic parameters, oxidative status and histopathological picture of ovaries. The effectiveness of letrozole in inducing PCOS in rats is well documented [28, 29]. It suppresses aromatase activity, thereby reducing the conversion of androgens to estrogens, leading to an excessive accumulation of androgens in the ovaries [30]. In PCOS, the gonadotropin-releasing hormone (GnRH) pulse rate rises, promoting the production of LH rather than FSH [31]. Therefore, increasing the concentration of LH will enhance androgen production in membrane cells, while the relative lack of FSH will reduce the potential of granulosa cells to convert androgens to estrogens and hinder maturation and ovulation of the follicles [32]. It is clear from this study that, PCOS rats have augmented testosterone and LH hormones levels when compared to control rats.

These results are consistent with Orio *et al.* [33], Palomba *et al.* [34], licorice extract and vit E succeeded in lowering testosterone and LH levels and the combination displayed the better effect than each one alone, this action may attributed to its estrogen like and antiandrogen activities. Licorice inhibits the activity of 17-hydroxysteroid dehydrogenase (17HSD) and 17-20 lyase while stimulating the activity of aromatase, according to several studies [35, 36]. Licorice can also influence the activity of 5a- and 5b-reductases [37]. Androgens and estrogens are synthesised and/or metabolised by all these enzymes, in addition, prior studies presented that vit. E can decrease testosterone level in PCOS [38].

This hormonal disorder of PCOS is accompanied by metabolic disorders including hyperglycemia, which is considered an important sign of PCOS. As Desai *et al*. [39] reported, this may have occurred due to high testosterone concentrations leading to insulin resistance. Insulin resistance is a physiological condition when the biological effects of insulin are lower than expected, cells do not respond to insulin, leading to disruption of glucose utilization and transmission [40, 41]. In addition to hyperglycemia, the lipid profile was imbalanced and dyslipidemia developed, dyslipidemia was attributed to hyperandrogenemia [42]. PCOS group in this experiment exhibited hyperglycemia and alteration in lipid profile where there was a significant elevation in triglycerides, total, LDL and VLDL cholesterol in comparison with control., licorice, vit.E and their combination restored the metabolic disorder generated by PCOS. Licorice has been shown to alter insulin resistance and lipid metabolism, through a variety of ways [10, 43, 44]. The following are some of licorice's potential mechanisms: 1) Increased fatty acid oxidation and control of lipid metabolism and lipolysis via impacts on gene expression in fatty acid production pathways [45], 2) Appetite Suppression because of its Strong Taste [46], 3) Activation of the PPAR gene [45] and 4) A reduction in intestinal fat absorption [47]. Moreover, Ebrahimi et al stated that vit. E can decrease triglycerides and cholesterol and improve insulin resistant occurred in PCOS patients [38].

Obviously, antioxidant enzymes in PCOS patients are reduced [48], in addition, letrozole-induced rat PCOS causes ovarian oxidative stress [49]. Estimation of oxidative stress biomarkers (such as MDA) and antioxidant biomarkers (such as TAC, SOD, GPx and glutathione (GSH)) are useful for studying the role of oxidative stress in disease pathogenesis, in addition, they also serve as a useful tool to assess hazards of oxidative damage [50]. In the current experiment the oxidative stress parameters of the PCOS group showed significant changes, in which a significant increase in MDA level was observed, but a decrease in GSH activity compared with the control, augmented formation of MDA may be due to the incremented oxidation of biomolecules resulting in excessive lipid peroxidation in membranes, proteins and genes. Elevated MDA levels are evidence of tissue deterioration caused by free radical-mediated mechanisms [51]. Licorice extract, vit. E and their combination results stated significant improvement in oxidative stress status of ovaries where, MDA level was decreased and the activity of reduced glutathione was increased versus PCOS, the combination of licorice and vit. E could restore the oxidative stress status of ovaries where; rats showed no significant difference versus the control ones. Antioxidants have been shown to improve oocyte maturation and have a favorable influence on embryo development in mice in several prior studies [52]. Ju *et al*. [53] also discovered that licorice flavonoids have strong antioxidant properties and can scavenge more free radicals than other antioxidants. Vitamin E is frequently employed in the field of reproductive medicine because it may effectively repair the negative effects of oxidative stress on the reproductive system. Within biological membranes, vitamin E is the most potent chain-breaking lipophilic antioxidant. It has the antioxidant characteristic of unsaturated fatty acids, which helps to maintain cell membranes and prevents biological damage [54]. Vitamin E supplementation was observed to prevent oocyte apoptosis in mice given orally with 60 mg/kg/day nicotine in one research [55]. Vitamin E is also reported to protect endothelial cells from apoptosis, which is triggered by substantial oxidative stress at the tissue level [56]. Female infertility, early delivery, miscarriage, eclampsia, fetal intrauterine development restriction and other pregnancy-related illnesses can all be caused by a shortage of vitamin E [57].

CONCLUSION

The current study found that a combination of licorice and vitamin E restored the reproductive and metabolic disorders that exhibited by PCOS, by reducing androgen aromatization and countering ovarian oxidative stress, which may be important in PCOS pathogenesis, in addition improving lipid profile and glucose level. As a result, licorice and vitamin E may be a promising choice for treating PCOS reproductive and metabolic issues.

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