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Hydroalcoholic extract of flaxseed improves polycystic ovary syndrome in a rat model

Gholamali Jelodar [1], Sajjad Masoomi [1], Farhad Rahmanifar [1]*

[1] Department of Basic Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

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ABSTRACT

Objective(s): Herbal medicines are an alternative choice for treatment or controlling of polycystic ovary syndrome (PCOS). Effect of hydroalcoholic extract of flaxseed was evaluated on ovarian hormones and histological changes of uterus and ovary in a PCOS-induced rat model.

Materials and Methods: Twenty four rats divided into four groups including negative control, positive control, PCOS and treatment groups. Positive control group received hydroalcoholic extract of flaxseed for 30 days. PCOS was induced by single intramuscular injection of estradiol valerate. Treatment group was treated with flaxseed extract 7 weeks after induction of PCOS for 30 days. Ovaries and uterus were dissected out and their sections were used for histomorphometric study. Levels of estradiol, progesterone, testosterone and dehydroepiandrosterone (DHEA) were measured in the serum.

Results: In the treatment group, flaxseed extract increased level of progesterone (P<0.05), while decreased testosterone (P<0.05) compared with the PCOS group. Concentrations of estrogen and DHEA did not change significantly in comparison with the PCOS group. Histomorphometric study showed that in the treatment group, the number of preantral follicles, antral follicles and corpus luteum increased compared with the PCOS group (P<0.05), but the number of cystic follicles and diameter of antral follicles decreased (P<0.05), and the number of primary follicle did not alter significantly. In the treatment group, the thickness of granulosa layer increased, but the thickness of theca layer and tunica albuginea decreased compared to the PCOS group (P<0.05).

Conclusion: Hormonal profile and histomorphometric features of ovary that were disturbed by PCOS induction were ameliorated by hydroalcoholic extract of flaxseed.

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Introduction

Polycystic ovary syndrome (PCOS) is a female endocrine disorder. Women suffering from this disease have infertility problems mostly because of hormonal imbalance. Patients often show elevated levels of androgens in plasma. Metabolic disturbances such as insulin resistance can activate hypothalamic-pituitary-adrenal (HPA) axis and result in the increased production of androgens in PCOS patients (1, 2).

A number of endocrine disorders that enhance and intensify each other may be observed in PCOS. These disorders include hypothalamic-pituitary-gonad (HPG) axis dysfunction. In fact, PCOS includes abnormal gonadotropin secretion and increased secretion of ovarian steroids. Luteinizing hormone (LH), particularly in women with PCOS, increases due to an increase in the frequency and secretion of these hormones. When the concentration of LH increases compared to follicle stimulating hormone (FSH), ovaries preferentially increase androgen synthesis (3). The National Institutes of Health postulates that higher concentrations of androgens in blood and/or clinical hyperandrogenism are the key criteria for PCOS diagnosis (4).

Various therapeutic methods such as changing of life habits, surgery and medication like clomiphene citrate, metformin, letrozole and tamoxifen have been proposed for PCOS (3). Today, herbal medicines are widely used as an alternative for treatment or controlling of diseases. In this regard, in a case study, reduced testosterone and increased insulin level was reported in a 31-years old woman with PCOS following treatment by flaxseed (*Linum usitatissimum*) (30 g/day for 4 months) (5). Administration of 15 g/day of flaxseed for 12 weeks to a 48 years old woman (postmenopausal) was also reported to reduce testosterone with no significant changes in estrone and estradiol level (6). Phytochemical screening of hydroalcoholic extract of flaxseed (7) has been presented in Table 1. Hence this study was designed to evaluate the effect of hydroalcoholic extracts of flaxseed on endocrine and histomorphometry changes of ovary and uterus in estradiol valerate-induced PCOS rats.

Materials and Methods

Animals and ethics

This experiment was accomplished under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986, regarding the standards in the protection of animals for experimental purposes were followed.

Twenty-four adult female Sprague Dawley rats, weighing approximately 200±20 g, were used. Animals were kept in standard polypropylene cages at 22±2 ˚C temperature, 38% relative humidity, and 12/12 hr

*Corresponding author: Farhad Rahmanifar. Department of Basic Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. Tel: +98-71-36138652; Fax: +98-7132286940; Email: f.rahmanifar@yahoo.com

Table 1. Phytochemical screening of hydroalcoholic extract of flaxseed (*Linux usitatissimum*) (7)

Name of the compound	Name of the test	Results*
Tannins	5% ferric chloride test	+
Flavonoids	Alkaline reagent test	-
Carbohydrates	Molischdr test	+
Phenols	Ferric chloride test	+
Alkaloids	Mayeroidslor	+
Steroids	Chloroform + acetic acid + H_2SO_4	+
Glycosides	Legalsi test	-
Proteins	Ninhydrin test	+
Diterpenes	Copper acetate test	+
Saponnins	Distilled water	-

*-, absent ; +, present

light/dark cycle, and fed with a standard pellet diet and water *ad libitum*.

Preparation of hydroalcoholic extract of flaxseed

Flaxseed was ground into a fine powder using a homogenizer and the powder was used for extraction with 70% ethanol as previously described (8). The extract was concentrated under reduced pressure in rotary evaporator to yield a crude semi-solid mass. The resultant semi-solid extract was then lyophilized to fine powder in a lyophilizer. It was then stored in airtight container at 4 ˚C.

Induction of PCOS and flaxseed treatment

A diagram demonstrating the study design is shown in Figure 1. Reproductive cycle of all animals was determined by vaginal smear for 14 days (9) (Figure 2). The rats with regular cycles were randomly divided into 4 equal groups (n=6). Negative control and positive control groups received one dose of intramuscular injection of 0.2 ml sesame oil (as solvent of estradiol valerate) at the start of the study. Furthermore, the positive control group was treated with 200 mg/Kg hydroalcoholic extracts of flaxseed orally by gavage for 30 days. PCOS was induced in PCOS group by intramuscular injection of estradiol valerate but was not treated following induction of PCOS. Treatment group, after induction of PCOS, was treated with 200 mg/Kg hydroalcoholic extract of flaxseed, orally by gavage, following induction of PCOS.

PCOS was induced in rats by single intramuscular injection of 4 mg of estradiol valerate, which is a long-acting estrogen. Administration of estradiol valerate causes dysregulation of gonadotropin-releasing hormone (GnRH), resulting in improper release and storage of LH. This hormone is considered a key pathogenic factor in the development of PCOS. A single dose of estradiol valerate to cyclic rat induces anovulation and polycystic ovaries within 7 weeks (10). The treatment with flaxseed was started 50 days after estradiol valerate injection in PCOS-induced treatment group and this was considered as the first day of treatment for 30 days. Reproductive

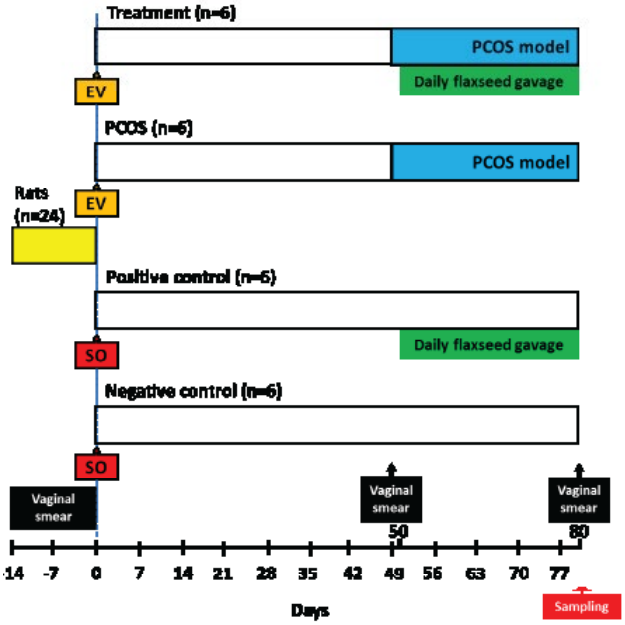

Figure 1. Schematic diagram of the experimental design including induction model, treatment, vaginal smear checking and sampling time for evaluation of the effect of hydroalcoholic extract of flaxseed on polycystic ovary syndrome (PCOS) in a rat model. EV, estradiol valerate; SO, sesame oil

cycle of animals was monitored after induction of PCOS on day 49 and before sampling on day 80 (Figure 1).

Blood and tissue samplings

On day 80 after model induction (Figure 1), whole blood was collected through heart puncture and their sera were used for hormonal measurement. Ovaries and uteri were then dissected out and subjected to histomorphometric study according to Rahmanifar *et al* (11).

For ovary and uterus histomorphometric evaluation, after fixation, segments were dehydrated in ethanol and xylene and then were embedded in paraffin wax, and histopathologic sections were made from each block. Serial sections of ovaries were prepared at thicknesses of 10 µm. The 5 µm thickness sections of uterus were

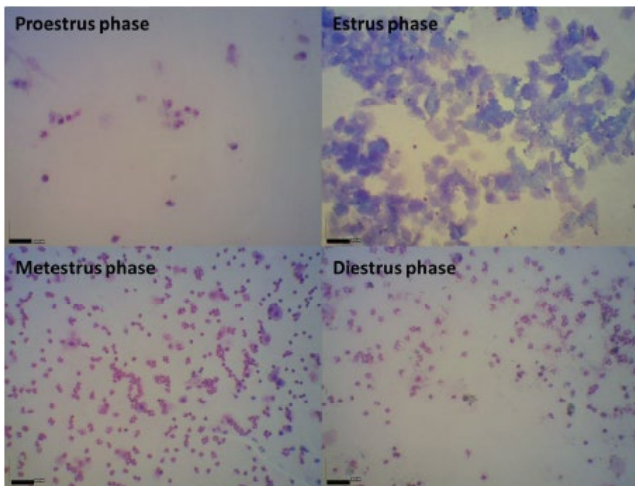

Figure 2. Vaginal smear of rats for detection of reproductive cycle. (Giemsa staining, index bars, 50 µm)

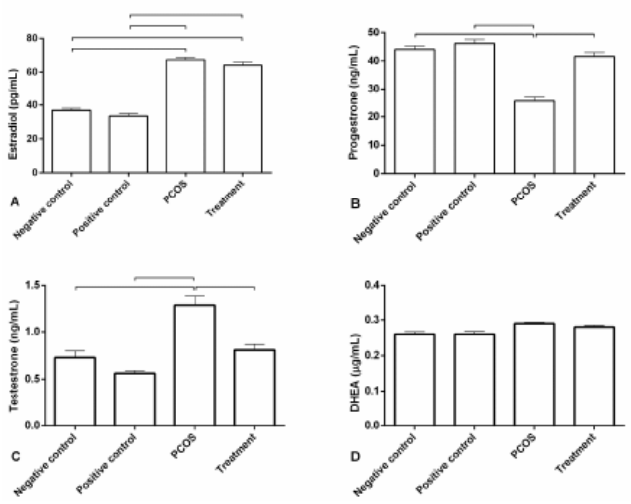

Figure 3. Effects of flaxseed extract on polycystic ovary syndrome (PCOS) rats and plasma concentrations of estradiol, progesterone, testosterone, and dehydroepiandrosterone (DHEA). Values presented as mean±SEM, the above lines indicate significant difference between groups

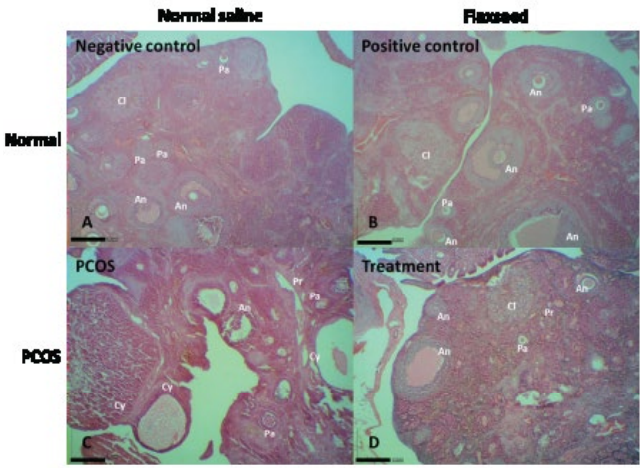

Figure 4. Survey views showing negative control (A), positive control (B), estradiol valerate-exposed rat (C), and flaxseed extract-treated rat (D) ovaries in the same magnification. Pr, primary follicle; Pa, preantral follicle; An, antral follicle; Cy, cystic follicle; Cl, corpus luteum (H&E staining; index bars, 200 µm)

also prepared. One section of every 10 serial sections of ovary and three sections of each horn of uteri were deparaffinized in 60 ˚C. Then, selected sections rehydrated in graded concentrations of xylene and ethanol. Finally, ovarian and uterine slices stained with hematoxylin and eosin.

To assess type of follicles (12), as well as counting primary, preantral, antral and/or cystic follicles and corpora lutea (13), ovarian slices were observed under a light microscope (CX21, Olympus, Japan) and were photographed by an adjusted digital camera (AM423U Eyepiece Camera, Dino-Eye, Taiwan). Tunica albuginea, granulosa layer, and theca layer thicknesses of follicles (11) and endometrial and myometrial thicknesses of the uterus (14) were measured by Dino Capture 2.0 software (AnMo Electronics Corporation, New Taipei City, Taiwan). Testosterone, progesterone, estrogen and dehydroepiandrosterone (DHEA) were measured in serum by ELISA method (Accu-Bind, Monobind Inc., Lake Forest, CA, USA) (15, 16).

Statistical analysis

The results are presented as means ± standard error of mean (Mean ± SEM). Statistical Package for Social Sciences (SPSS-16.0) were used and the results were analyzed using one-way analysis of variance (ANOVA) followed by *post hoc* multiple comparisons Tukey test for comparison between different treatment groups. Statistical significance was set at $P<0.05$.

Results

Flaxseed extract improved serum sex steroids in PCOS

Level of estradiol in the PCOS and treatment groups increased compared to the positive and negative controls ($P<0.05$, Figure 3A). While, there was no significant difference in estradiol level between the PCOS and treatment groups. Progesterone concentration in the PCOS group decreased compared to the control and treatment groups ($P<0.05$, Figure 3B); however, the level of progesterone in the treatment group did not have significant difference with the control groups ($P>0.05$). In addition, mean testosterone level in the PCOS group was higher than the control and treatment groups ($P<0.05$, Figure 3C). On the other hand, there was no significant difference in testosterone level between the control and treatment groups. There was no significant difference between the groups regarding the level of DHEA ($P<0.05$, Figure 3D).

Flaxseed extract corrected ovarian histomorphology in PCOS

Light microscopic analysis of the negative and positive control groups showed no structural abnormalities in different stages of development and regression of follicles and corpus luteum, and no cystic follicles (Figures 4A and B). The area of the largest follicle was greater in the PCOS rats than the controls; a trend toward follicular enlargement was not observed in the treatment group (Figures 4C and D). The number of cystic follicles was increased in the PCOS group. In addition, the PCOS rats had no corpus luteum (Figure 4C). The theca and granulosa cell layers were normal in the control groups (Figures 5A and B). A large fluid-filled cyst was characteristic of cystic follicle with compressed granulosa cell layer and enlarged theca interna cell layer (Figure 5C). The cystic follicles' walls were thickened, because of thickened theca cell layer; while, this phenomenon was not observed after treatment with flaxseed extract (Figure 5D).

Flaxseed extract attenuated ovarian histomorphometric indices in PCOS

Numbers of preantral follicles, antral follicles and corpus luteum decreased in the PCOS group compare to the control groups ($P<0.05$, Table 2). Treatment by flaxseed extract increased their number and except the number of corpus luteum, other parameters returned to normal range ($P<0.05$). Histomorphometric evaluation of ovaries revealed that thickness of granulosa and tunica albuginea increased, while thickness of theca layer deceased in the PCOS group compare to the control

Table 2. Comparison of the number of ovarian follicles and corpus luteum after treatment of polycystic ovary syndrome (PCOS) in rat with flaxseed extract

Groups	Follicles				Corpus luteum
	Primary	Preantral	Antral	Cystic	
Negative control	19.37±1.15[a]	26.87±2.29[a]	10.0 ±0.65[a]	0[a]	10.93±0.54[a]
Positive control	21.57±1.42[a]	34.55±1.83[b]	11.35±0.89[a]	0[a]	11.25±0.93[a]
PCOS	10.41±1.54[b]	10.2±1.35[c]	2.80±0.42[b]	5.26±0.04[b]	2.60±0.21[b]
Treatment	13.55±0.58[b]	23.38±1.61[a]	8.55±0.73[a]	2.31±0.07[c]	6.45±0.82[c]

Values presented as mean±SEM. a,b,c different superscript letters in each column show significant difference observed between groups

Table 3. Histomorphometric comparisons of tunica albuginea and antral follicles layers after treatment of polycystic ovary syndrome (PCOS) in rat with flaxseed extract

Groups	Tunica albuginea thickness (µm)	Granulosa layer thickness (µm)	Theca layer thickness (µm)	Antral follicle diameter (µm)
Negative control	14.17±0.83[a]	53.63±2.89[a]	22.19 ±1.64[a]	556.27 ±14.18[a]
Positive control	13.69±0.74[a]	56.55±2.40[a]	19.73±2.19[a]	532.43 ±19.25[a]
PCOS	22.55±1.31[b]	31.67±1.62[b]	34.26±1.24[b]	703.36±8.51[b]
Treatment	17.37±1.38[c]	51.23±3.54[a]	24.36±1.84[a]	576.46±18.31[c]

Values presented as mean±SEM. a,b,c different superscript letters in each column show significant difference observed between groups

Table 4. Histomorphometric comparisons of uterine tissue layers after treatment of polycystic ovary syndrome (PCOS) in rat with flaxseed extract

Groups	Endometrium thickness (µm)	Myometrium thickness (µm)
Negative control	283.54±26.43	137.67±31.11
Positive control	305.14±27.68	121.45±32.67
PCOS	262.71±25.97	121.91±28.02
Treatment	293.32±24.49	115.45±26.56

Values presented as mean±SEM. No significant difference observed between groups

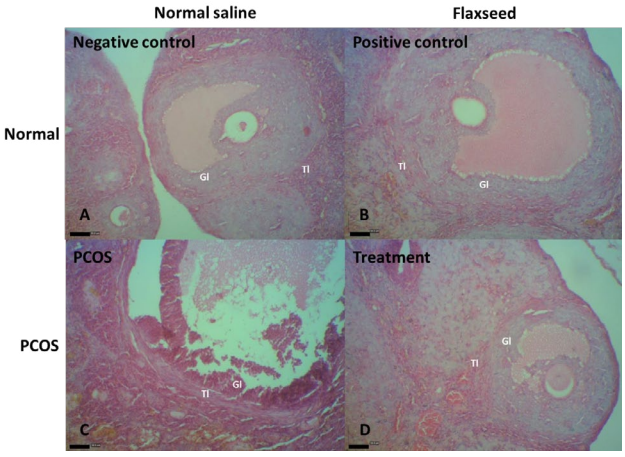

Figure 5. A and B, healthy tertiary follicle in the negative and positive control rats. The theca layers and granulosa layers appear normal. C, A follicle in the early process of atresia with apoptotic granulosa cells, most of which are in the inner parts of the granulosa layer in a polycystic ovary syndrome (PCOS) rat. Thin and elongated epithelioid cells form the inner surface of the wall. The cyst fluid contains macrophages. D, Ovary from a flaxseed-treated PCOS rat with normal tertiary follicles. Gl, granulosa layer; Tl, theca layer (H&E staining; index bars, 50 µm)

groups; however, consumption of flaxseed extract altered this value toward normal level (P<0.05, Table 3). No significant difference was observed in thickness of endometrial and myometrial layers of uteri between the

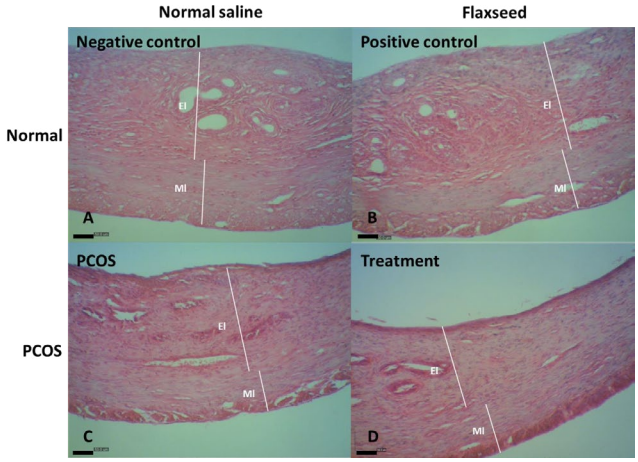

Figure 6. Uterine tissue sections of negative control (A), positive control (B), estradiol valerate-exposed rats (C), and flaxseed extract-treated (D) rats. El, endometrial layer; Ml, myometrial layer (H&E staining; index bars, 50 µm)

groups (P>0.05, Figure 6 and Table 4).

Therefore, flaxseed extract attenuated PCOS signs that were induced by estradiol valerate in rats. In addition, sex hormonal profile and histomorphometry of ovary that were disturbed by PCOS induction were ameliorated by flaxseed.

Discussion

Treatment of PCOS with flaxseed extract significantly deceased level of testosterone in the treatment group compare to the PCOS group with no significant difference with the control groups. Similar to our findings, in a case study on a 31-year-old woman with PCOS, it was reported that flaxseed supplementation of 30 g/day for 4 months reduced testosterone levels (5). This effect may be due to the negative feedback of flaxseed extract on LH. Treatment of the PCOS rats with flaxseed extracts had no significant changes on level of estradiol. Consistent with

our findings, in a clinical trial on 48 postmenopausal women, flaxseed extract did not change estrone and estradiol levels (6). Flaxseed as an herbal medicine can be considered for the management of women with PCOS and hyperandrogenism. Although no exact mechanism of the effect of flaxseed in PCOS has been reported (17), but the effects of flaxseed on menstrual regulation (18, 19) and hormonal concentration in postmenopausal women (19-21) has been reported.

In the present study, we used estradiol valerate for induction of PCOS. Following induction of PCOS, estradiol and testosterone were significantly increased, while level of progesterone significantly decreased in this group; however, no significant change in levels of DHEA was observed. Similar results were also reported by other researchers following induction of PCOS by estradiol valerate (22, 23). Estradiol valerate (2 mg) in adult rat or estradiol benzoate (0.5 mg) in neonatal rat (0 to 2 days) induced PCOS reproductive features with a single subcutaneous injection (24, 25). Two months later, polycystic ovaries of induced rats exhibited elevation of testosterone and estradiol levels (24). Increase in the secondary follicle population, ovarian theca-interstitial area, and expression of LH receptor and *Cyp17a1* genes are induced by estrogen (25). Moreover, pre-pubertal rats subcutaneously implanted with 10 mg diethylstilbestrol showed follicle growth, without luteinizing the follicles and therefore can be served as another model of PCOS (26). Also in mice, pre-pubertal estrogen stimulation led to follicular development synchronization, but no typical symptoms of PCOS remained at adulthood (27). Physiology and anatomy of the ovary PCOS in estradiol model resemble those of human. In addition, inconsistent characteristics of sex hormone profile and ovarian morphology of estrogen models in different studies have been reported (10, 22, 28-34).

Regarding to ovarian histomorphology, induction of PCOS resulted in significant reduction in the number of primary, preantral and antral follicles compared to the negative control group, while treatment with flaxseed improved the condition. In other words, flaxseed is somewhat effective in the growth and development of follicles, and corpus luteum and reduces the cyst follicles after induction of PCOS. Consistent with our results, induction of PCOS in female rats with estradiol valerate led to decrease of the average number of primary, secondary and Graffian follicles compared to the normal control group (35). Increase in the number and growth of Graffian follicles in immature female rats was reported following treatment with flaxseed extract (36). Morphological study of ovaries showed a significant decrease in the number of granulosa cell layers around the cystic follicles compared to the control group. The HPG axis dysfunction in PCOS may lead to the increase of GnRH, and improper secretion of LH and FSH causes disruption of ovarian function and growing of more preantral follicles, which may not convert to antral follicles (37).

No significant difference in the average of endometrium and myometrium thickness was observed between study groups that are in contrary with previous report, which indicated increase in the height of the epithelium and decrease in the thickness of the endometrium in rats following induction of PCOS with estradiol valerate (38).

Conclusion

Findings of the current study showed that hydroalcoholic extract of flaxseed attenuated PCOS signs in rats. Although, estradiol valerate model of PCOS did not show all phenomena of human PCOS, but sex-steroid hormonal profile and histomorphometric characteristics of ovary in treated rats were ameliorated by flaxseed. Although, in a recent clinical trial, flaxseed oil omega-3 supplementation in PCOS women for 12 weeks had beneficial effects on some metabolic status (39), but further studies in PCOS women needs to confirm therapeutic effects of hydroalcoholic extract of flaxseed.

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