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Research article

Hydroalcoholic extract of *Glycyrrhiza glabra* root combined with *Linum usitatissimum* oil as an alternative for hormone replacement therapy in ovariectomized rats

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ABSTRACT

Objective: Plant-derived estrogens (phytoestrogens) with structural similarity to primary female sex hormones could be suitable replacements for sex hormones. Therefore, the effects of the licorice root extract and *Linum usitatissimum* oil on biochemical and hormonal indices in the serum and uterine stereological changes in ovariectomized rats were evaluated.

Design: In this study, 70 adult female rats were randomly divided into seven groups including 1) control group, 2) sham-operated group, 3) ovariectomized (OVX) group, 4) OVX rats that received 1 mg/kg estradiol for 8 weeks at the day of post-operation, 5) OVX rats which received 2.0 mg/kg body wt *Linum usitatissimum* oil for 8 weeks at the day of post-operation, 6) OVX rats which received 2.0 mg/kg body wt licorice extract for 8 weeks at the day of post-operation, and 7) OVX rats which received 2.0 mg/kg body wt *Linum usitatissimum* oil + 2.0 mg/kg body wt licorice extract for 8 weeks at the day of post-operation. After eight weeks, alkaline phosphatase activity, as well as calcium, estradiol, and progesterone concentrations were assessed and tissue samples of the uterus were serologically examined.

Results: The results indicated that after 8 weeks of OVX the alkaline phosphatase activity (Mean = 637.7 IU/L) increased and the calcium (Mean = 7.09 mg/dl), estradiol (5.30 pmol/L), and progesterone (Mean = 3.53 nmol/L) reduced compared to other groups. Moreover, stereological changes in the uterus in ovariectomy groups were seen compared to the other groups. The treatment with *Linum usitatissimum* oil and licorice extract had a significant therapeutic effect on biochemical factors and stereological changes compared to the ovariectomized group.

Conclusion: The results of this study showed that the combination of *Linum usitatissimum* oil with licorice extract showed the high potential of hormone replacement therapy in the reduction of OVX complications.

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1. Introduction

Oophorectomy or ovariectomy (OVX) is the surgical removal of one or both ovaries usually offered in women with ovarian cancer (ranks fifth in cancer deaths), severe endometriosis, cysts, and torsion of the ovary [1–3]. In the US, 23% of women aged 40–44 years and 45% of women aged 45–49 years have concomitant elective oophorectomy to prevent the subsequent development of ovarian cancer [4]. In another study, it was shown that 55% of all women and 78% of women between 45 and 64 years of age had bilateral oophorectomy at the time of a hysterectomy, suggesting that approximately 300,000 US women have prophylactic bilateral oophorectomy at the time of hysterectomy every year [5]. Although limited data is available related to the cost-effectiveness of oophorectomy in long term in society, generally speaking, the potential cost reduction for the healthcare system of € 791,653 was seen if risk-reducing (bilateral) salpingo-oophorectomy had been performed before the development of cancer [6].

The removal of ovaries disrupts the hormonal and menstrual cycle in women. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen, and progesterone are the four main hormones, affecting the menstrual cycle. Estrogen is also an important immune modulator that enhances B-cell activity to produce more antibodies and improve survival from infection [7,8]. Estrogen and progesterone enhance serotonin and histamine release and stimulate fibroblast activities and nerve growth. Estrogen also has physiologic effects on the breast, bone, skin, muscles, liver, brain, and lipid metabolism [9]. Loss of ovarian hormones following OVX elevates the risks of cognitive impairment and dementia and results in an increased risk of diabetes, obesity, stroke, and osteoporosis as well as genitourinary and sexual dysfunction [10,11]. During periods of estrogen and progesterone withdrawal (OVX, postpartum, and menopause), there are often clinical rebounds and an increase in disease flares with the release of T-cell suppression, causing osteoporosis and rheumatoid arthritis [12]. Accordingly, under normal conditions, women have to spend about a third of their lives with a decrease or lack of estrogen and ovarian hormones [13], and long-term use of estrogen increases the risk of endometriosis and breast cancer [14,15]; researchers are looking for a good alternative therapy to take advantage of estrogen without its side effects.

Hormone Replacement Therapy (HRT) was proposed as a suitable therapeutic approach in ovariectomized and postmenopausal women [16]. Noteworthy, estrogen replacement reduces coronary diseases, osteoporosis, and genital atrophy [17]. The postmenopausal woman who is not on HRT experiences progressive atrophy of vaginal epithelium, a change in vaginal pH, a decrease in the quantity of vaginal secretions, and a decrease in the general circulation to the vagina and uterus [18].

Meanwhile, plant estrogens (phytoestrogens) are the focus of studies [19]. Phytoestrogens with similar estrogenic structures can bind to estrogen receptors [20] and mimic the action of estrogens with significantly fewer side effects compared to industrial estrogens [21]. The estrogenic strength of these compounds is estimated at one-thousandth to ten-thousandth of estradiol [22].

Overproduction of reactive oxygen and nitrogen species (ROS and RNS) induces DNA damage, endothelium degradation, and granulosa cell apoptosis, all of which are deleterious to the ovarian and reproduction tissues. As a result, antioxidants may be able to counteract the negative effects of free radicals on reproductive organs [23,24].

Glycyrrhiza glabra (licorice) belongs to the Fabaceae family. This species is native to Mediterranean areas, but it is also present in India, China, and Iran [25]. Licorice has a long story in traditional medicines and folk remedies to treat gastrointestinal problems, dyspepsia, inflammation, and arthritis. According to the World Health Organization, licorice is employed as a demulcent in the treatment of sore throat and bronchial catarrh, confirming the low toxicity of the mentioned natural product [26]. Licorice is a rich source of proteins, amino acids, polysaccharides, simple sugars, mineral salts, phytoestrogens, tannins, phytosterols (sitosterol and stigmasterol), coumarins, and vitamins (B1, B2, B3, B5, E, and C) [27], some of which have antioxidant, anti-inflammatory, antiviral, and antimicrobial properties. Licorice has also been proven to have anti-ulcer activities in gastric mucosal injury, ulcerative colitis, and injured tissue, as well as hepatoprotective, antiproliferative, and anticancer properties. Other biological effects such as skin whitening, depigmentation, antiaging, and memory enhancement have also been documented [28,29]. However, excess licorice intake is associated with reactions of cardiovascular system complications [30,31].

Flaxseed, *Linum usitatissimum*, is also known as linseed. *Linum usitatissimum* and its bioactive components have been frequently used as dietary supplementation for their beneficial activities on cardiovascular disease, inflammation, atherogenesis, lipid profile, arrhythmias, hypertension, oxidative stress, and estrogen activity [32,33]. *Linum usitatissimum* oil is one of the richest sources of ω-3 (*n*-3) polyunsaturated fatty acids (n-3 PUFA), especially α-linolenic acid (ALA) followed by n-6 PUFA linoleic acid. Studies confirmed that the beneficial effects of oil were due to the presence of essential fatty acids as well as lignans, cyclic peptides, cyanogenic glycosides (CGs), flax proteins, and soluble and insoluble fiber [34]. Controlled experimental diets of *Linum usitatissimum* have demonstrated numerous beneficial effects including hepatoprotective, anti-inflammatory, antiulcerogenic, cardio-protective anticancer, antitumor multiplicity, and hormone supplement properties [35,36]. Also, consumption of *Linum usitatissimum* in patients with pulmonary artery disease with elevated blood pressure significantly reduced both systolic and diastolic blood pressure [37]. Flaxseed consumption is also associated with a reduction in serum TG and LDL cholesterol without any alteration of high-density lipoprotein (HDL) [38], suppressing the development of atherosclerosis [39,40]. *Linum usitatissimum* protected the kidney against RIR-induced renal injury, probably by inhibiting ROS [41]. However, taking 6 g per day of *Linum usitatissimum* oil was associated with stomach and intestinal discomfort in adults [42]. Some allergic reactions to *Linum usitatissimum* were also reported [43].

The aim of this study was to investigate the efficacy of *Linum usitatissimum* oil (due to containing high amounts of PUFA) and licorice root extract (consisting of antioxidants, phytoestrogens, and good taste) on menopause symptoms in ovariectomized rats. In this context, the alkaline phosphatase activity (ALP), Ca ions, estradiol, and progesterone concentrations, as well as uterine stereological change including the uterus weight and volume, and the volume of the uterine perimetrium layer, myometrium layer, endometrium layer was determined. Other factors include the average volume of the uterine glands, the average thickness of the uterine perimetrium layer, the average thickness of the uterine perimetrium layer, the average diameter of the uterine tissue vessels, and the

average thickness of the uterine endometrium layer were also recorded.

2. Materials and methods

2.1. Ethical considerations

Experimental research on plants was following international legislation and guidelines of the Department of Pharmacognosy, Shiraz University of Medical Sciences, Shiraz, Iran. Ethical approval was confirmed by the Animal Care Committee of Shiraz University of Medical Sciences (IR.SUMS.REC ethical code: 90-01-67-3774). The authors followed up all institutional and international guidelines for animal care during this study. Also, the work has been reported in line with the Animal Research Reporting *in vivo* Experiments guidelines (ARRIVE).

2.2. Providing Linum usitatissimum oil and hydroalcoholic extract of licorice root

Linum usitatissimum oil was purchased from Zardband Pharmaceuticals company (https://zardband.com/en/) in which the *Linum usitatissimum* oil was obtained through a cold press of seed under 50 °C so that the natural properties of the oil were preserved. Licorice roots were collected from Abadeh area, a region in the north of Fars province, Iran. The licorice root (voucher number: MPH-2670-1) was dried and ground. Then, the powder was incubated in a percolator using 500 mL of ethanol 70% for 72 h. The extra solution was vaporized by the rotary to concentrate the extract and kept at −20 °C until use [46,47].

2.3. Rats groups

Seventy mature females Sprague Dawley aged 6 months and weighing between 200 ± 20 g were purchased from the laboratory animals center of Shiraz University of Medical Sciences. The rats were maintained under standard housing laboratory conditions with a relative humidity of 60 ± 5%, a temperature of 23 ± 2 °C, and 12-hr light/dark cycles in housing cages with the dimension of 42 cm length × 26.5 cm width × 15 cm height in which five rats were kept in each cage. Rats were fed with a standard pellet diet and water ad libitum. After 1 week of adaptation to the diet and the new environment, the animals were screened. Before ovariectomy, vaginal smears were collected from all animals for 2 weeks to evaluate the regularity of their reproductive cycle using microscopic observation. The OVX was done during the estrus phase of the estrous cycle [48,49]. The rats were divided into several groups (n = 10), as shown in Table 1.

2.4. Preparation of treatment

2.0 mg/kg body wt *Linum usitatissimum* oil was dissolved in almond oil or 2.0 mg/kg body wt hydroalcoholic extract of licorice root was dissolved in normal saline; the prob was sonicated for 1 min on ice to properly disperse and dissolve the active compounds. Next, for the oral administration, the gavage technique with biomedical needles (length 76.2 mm, diameter 3 mm, straight) was applied and forced the animals to swallow the treatment.

2.5. Ovariectomy, blood sampling, and obtaining uterus tissues

Finding an ideal model to study OVX is difficult; however, it is proposed that the rat model has a similar hormonal function, ovarian aging, and changes to the menopausal transition in women. Also, rats have multiple features and endocrine changes like women including a decline in follicles, irregular cycling, steroid hormone fluctuations, and irregular fertility [50,51]. As a result, to develop the OVX model, rats were anesthetized with ketamine 10% (70–100 mg/kg) and xylazine 2% (5–10 mg/kg). The position of surgery was sterilized *via* betadine 10%. After ligation of the uterine horn through a midline longitudinal incision, both ovaries were surgically removed in all the groups, except for the control and sham groups. The sham-operated rats had their ventral incision, and the

Table 1
Rats groups.

No.	Group name	Operation
1.	Control group	No surgical operation + no OVX + received normal saline for 8 weeks at the day of post-operation.
2.	Sham group	Surgical operation + no OVX + treated by almond oil (2.0 mg/kg) for 8 weeks at the day of post-operation.
3.	Ovariectomy (OVX) group	Surgical operation + OVX + treated by almond oil (2.0 mg/kg) for 8 weeks at the day of post-operation.
4.	Experimental group 1 (OVX + Estradiol)	Surgical operation + OVX + treated by estradiol (1 mg/kg) dissolved in almond oil for 8 weeks at the day of post-operation.
5.	Experimental group 2 (OVX + Lin)	Surgical operation + OVX + treated by 2.0 mg/kg body wt *Linum usitatissimum* oil dissolved in almond oil for 8 weeks at the day of post-operation.
6.	Experimental group 3 (OVX + Gly)	Surgical operation + OVX + treated by 2. 0 mg/kg body wt hydroalcoholic extract of licorice root dissolved in normal saline for 8 weeks at the day of post-operation.
7.	Experimental group 4 (OVX + Lin + Gly)	Surgical operation + OVX + treated by 2.0 mg/kg body wt *Linum usitatissimum* oil dissolved in almond oil and 2.0 mg/kg body wt hydroalcoholic extract of licorice root dissolved in normal saline for 8 weeks at the day of post-operation.

manipulation of ovaries was performed without excising them [52]. Based on the group, the treatment started at the day of post-operation and all rats were fed similarly [53]. At the end of the study, rats were anesthetized with ketamine and xylazine and 2 ml of blood from each rat was collected *via* cardiac puncture procedure with the syringe. After collection of the blood, allow the blood to clot by leaving it undisturbed at room temperature. The clot was removed by centrifuging at 3000×*g* for 4 min in a refrigerated centrifuge. The resulting supernatant is designated serum. Finally, the rats were euthanized with a rapid and humane method using a 30% volume displacement rate of CO_2 increased to 70% in the induction chamber, and ovaries were removed.

2.6. Biochemical assays

The biochemical assessment was performed after eight weeks of treatment. The concentration of Ca ions and activity of alkaline phosphatase enzyme (ALP) were evaluated in serum samples by Biosystem SA (Spain) assay kit and atomic absorption methods, respectively [54,55]. Estradiol and progesterone levels were also quantified by enzyme-linked immunosorbent assay (ELISA) analytical biochemical technique [56]. The results were obtained by independent specialists who were blinded to the group allocations.

2.7. Stereological studies

The volume of the uterus was assessed *via* Cavalieri method [57]. In this method, using uniform and parallel random incisions and with a certain distance, the sample size was calculated by holding the area of the incisions and the distance between them. Based on the Cavalieri method, from each tissue sample, between 8 and 12 incisions were examined. The estimated volume was calculated using estV = t. (A1+A2+ … +Am) formula. The t, m, and A show the average thickness, numbers of random incisions, and area, respectively. For calculating the area (A), a point network that was randomly placed on the surface of the incision was used. Then, the calculation of volumetric ratios of different layers of uterine tissue (endometrium, myometrium, and perimeter) was done by the method of counting points and according to the Delesse ($Vv = \frac{P(structure)}{P(reference)}$) formula. P (structure) indicates the number of points that have collided with different layers, and P (reference) represents the total number of points that collided with the total uterine tissues. Therefore, the absolute volume of the layers was calculated by multiplying the volume ratio by the total volume.

To estimate the thickness of the different layers of the uterus, a probe with parallel lines and equal distances (Orthogonal Intercepts Probe) was. Therefore, to estimate the thickness of the layers, the desired probe was placed on the tissue; then, from the parts where the probe lines collided with the surface of the desired layer, the shortest linear path was drawn perpendicular to the other surface of the layer and its length was measured. However, since the thickness in different parts of the desired layer might be different, the following formula was used to eliminate this error: $T = \frac{\pi}{4} \times L_0$. T indicates the estimated thickness and L_0 represents the average measured thickness [58].

2.8. Statistical analysis

The raw numbers obtained from biochemical, hormonal, and stereological studies were analyzed using SPSS statistical software (Version 19.0, Chicago, IL, USA). The one-way ANOVA method and Tukey post hoc test were used to analyze and compare the results. The results were presented as mean ± standard deviation, and the significance level was set as $p \leq 0.05$.

3. Results

3.1. Biochemical assays

3.1.1. Alkaline phosphatase activity (ALP)

There was a significant increase in the activity of the ALP enzyme in the OVX group (Mean = 637.7 IU/L) compared to the control (Mean = 162.5 IU/L) and sham groups (Mean = 163 IU/L) ($F(6,63) = 47.177$, $P < 0.001$). Also, in all OVX-treated groups, ALP activity showed a significant decrease compared to the OVX group ($F(6,63) = 47.177$, $P < 0.05$), so the decrease in the groups which received estradiol (Mean = 164.1 IU/L) and *Linum usitatissimum* L. oil + licorice root extract (Mean = 190.1 IU/L) was equal to the control group and did not show a significant difference with it. Although, there was a significant increase of ALP in the groups which just received *Linum usitatissimum* oil (Mean = 271.8 IU/L) and licorice root extract (Mean = 283.1 IU/L), compared to the control group in which *P* values were 0.049 ($F(6,63) = 47.177$) and 0.020 ($F(6,63) = 47.177$) respectively. The results are shown in Fig. 1.

3.1.2. Ca ions concentrations

As depicted in Fig. 2, there was a significant decrease in the Ca^{2+} ions concentrations in the OVX group (Mean = 7.09 mg/dl) compared to the control (Mean = 10.58 mg/dl) and sham groups (Mean = 10.29 mg/dl) ($F(6,63) = 15.001$, $P < 0.01$). On the other hand, in all the OVX-treated groups, the concentration of Ca ions showed a significant increase compared to the OVX group ($F(6,63) = 15.001$, $P < 0.05$), so the increase in the groups which received estradiol (Mean = 9.88 mg/dl), licorice root extract (Mean = 10.02 mg/dl) and *Linum usitatissimum* oil + licorice root extract (Mean = 10.31 mg/dl) was equal to the control group and did not show a significant difference in the mentioned groups.

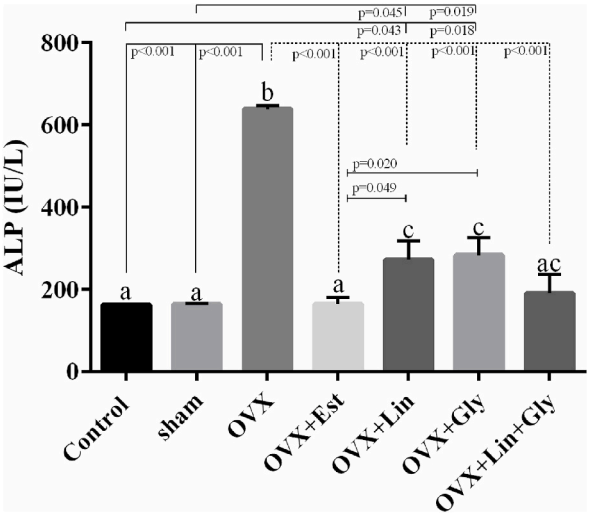

Fig. 1. The activity of ALP in the studied groups. The dots are shown as Mean ± SD. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). One-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

3.1.3. Estradiol concentrations

There was a significant decrease in the concentration of estradiol (Fig. 3.) in the OVX group (Mean = 5.30 pmol/L) compared to the control (Mean = 20.65 pmol/L) and sham (Mean = 19.12 pmol/L) groups ($F(6,63) = 49.798$, $P < 0.001$). On the other hand, in all the OVX-treated groups, estradiol concentration showed a significant increase compared to the OVX group ($F(6,63) = 49.798$, $P < 0.05$), so the increase in the groups which received estradiol (Mean = 19.24 pmol/L) and *Linum usitatissimum* oil + licorice root extract (Mean = 19.79 pmol/L) was equal to the control group and did not show a significant difference between them. However, in the single treatment such as licorice root extract (Mean = 11.82 pmol/L) and *Linum usitatissimum* oil (Mean = 13.41 pmol/L) groups, estradiol concentration showed a significant difference between the control and sham-operated groups ($F(6,63) = 15.001$, $P < 0.001$).

3.1.4. Progesterone concentrations

Progesterone levels in the experimental groups are shown in Fig. 4. There was a significant decrease in the concentration of progesterone in the OVX group (Mean = 3.53 nmol/L) compared to the control (Mean = 11.52 nmol/L) and sham (Mean = 10.78 nmol/L) groups ($F(6,63) = 77.705$, $P < 0.001$). On the other hand, in all the OVX-treated groups, the concentration of progesterone showed a significant increase compared to the OVX group ($F(6,63) = 77.705$, $P < 0.05$). Interestingly, the *Linum usitatissimum* oil +

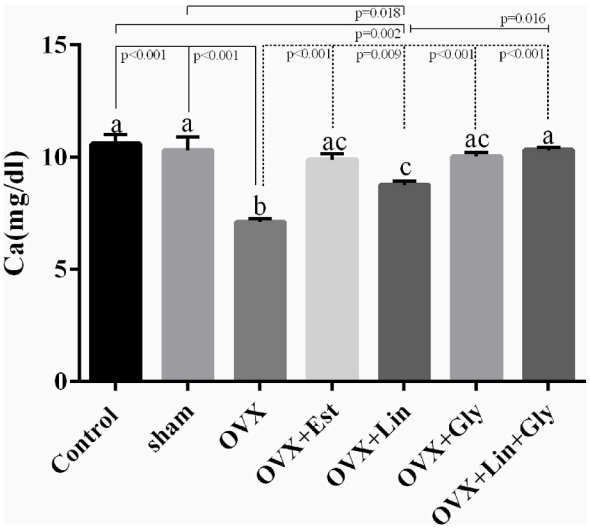

Fig. 2. The concentrations of Ca ions in the studied groups. The dots are shown as Mean ± SD. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). One-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

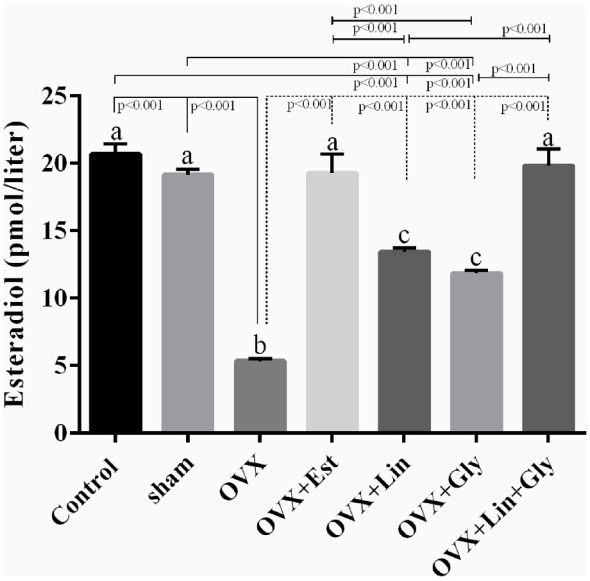

Fig. 3. The concentrations of estradiol in the studied groups. The dots are shown as Mean ± SD. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). One-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

licorice-treated group (OVX + Lin + Gly rats group with Mean = 11. 6.59 nmol/L) showed even more increase in the progesterone levels compared to OVX + Lin (Mean = 6.50 nmol/L) and OVX + Gly (Mean = 5.69 nmol/L) groups although the change was not significant.

3.2. Stereological studies

3.2.1. Effect of treatments on the uterus weight and volume

As depicted, ovariectomy (OVX group) caused a significant decrease in the uterus weight (Fig. 5, **a**) and volume (Fig. 5, **b**) compared to the control and sham groups ($F(6,63) = 29.483$, $P < 0.001$). On the other hand, OVX + Est and OVX + Est + Gly groups showed a significant increase in the mean weight and volume of uterine compared to the OVX group ($F(6,63) = 29.403$, $P < 0.001$) with no significant difference in the control and sham groups.

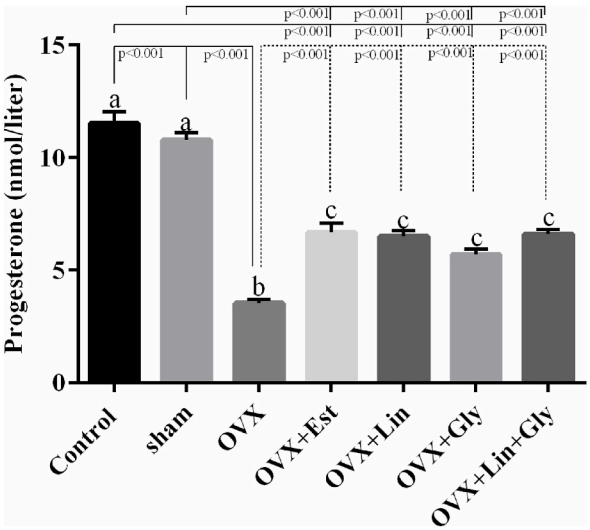

Fig. 4. The concentrations of progesterone in the studied groups. The dots are shown as Mean ± SD. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). One-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

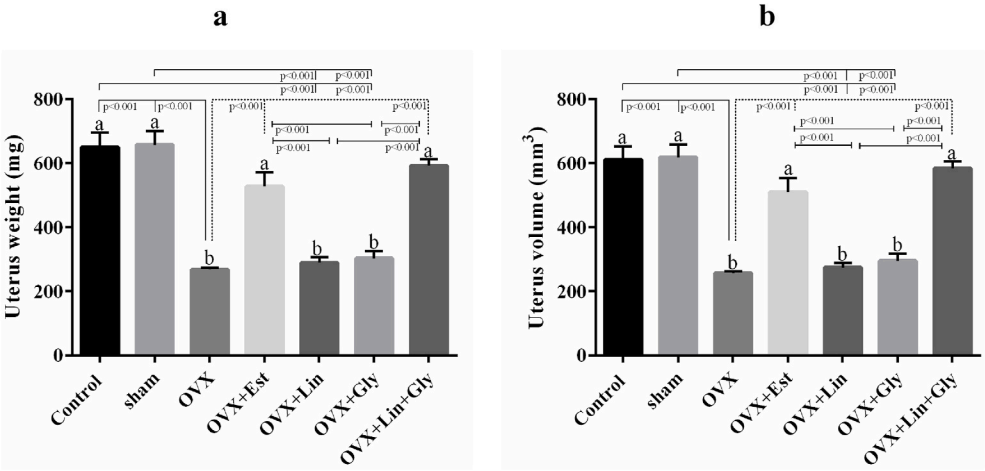

Fig. 5. The average weight (**a**) and total volume of the uterus without a uterine horn (**b**) in the studied groups. The dots are shown as Mean ± SD. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). The one-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

3.2.2. *The average volume of the uterine perimetrium layer*

A significant decrease in the mean volume of the uterine perimetrium layer was observed (Fig. 6) in the OVX group (Mean = 4.49 mm^3) compared to the control (Mean = 8.75 mm^3 with $F(6,63) = 6.302$, $P = 0.011$) and sham (Mean = 8.77 mm^3 with $F(6,63) = 6.302$, $P = 0.019$) groups. On the other hand, in estradiol (Mean = 8.45 mm^3 with $F(6,63) = 6.302$, $P = 0.029$) and *Linum usitatissimum* oil + licorice root extract (Mean = 8.48 mm^3 with $P = 0.030$) receiving groups, the mean volume of the uterine perimetrium layer showed a significant increase compared to the OVX group. There was no significant difference in the groups which received *Linum usitatissimum* oil and licorice root extract alone compared to the OVX group; in other words, the mean volume of the premium layer decreased significantly in these two groups compared to the control and sham groups ($F(6,63) = 6.302$, $P < 0.01$).

3.2.3. *The average volume of the uterine myometrium layer*

According to Fig. 7, a significant decrease in the mean volume of the uterine myometrium layer was observed in the OVX group (Mean = 72.7 mm^3). On the other hand, in the estradiol-treated group (Mean = 217.0 mm^3) and *Linum usitatissimum* oil + licorice root extract-treated group (Mean = 203.9 mm^3), the mean volume of the uterine myometrium layer showed a significant increase compared to the OVX group ($F(6,63) = 37.971$, $P < 0.001$). *Linum usitatissimum* oil administration (Mean = 77.7 mm^3) and licorice root extract (Mean = 74.2 mm^3) could not improve the mentioned parameters compared with the OVX control.

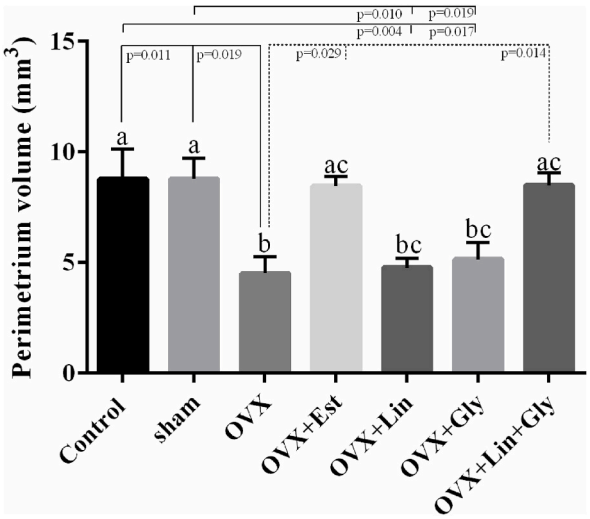

Fig. 6. The average volume of the uterine perimetrium layer in the studied groups. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). One-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

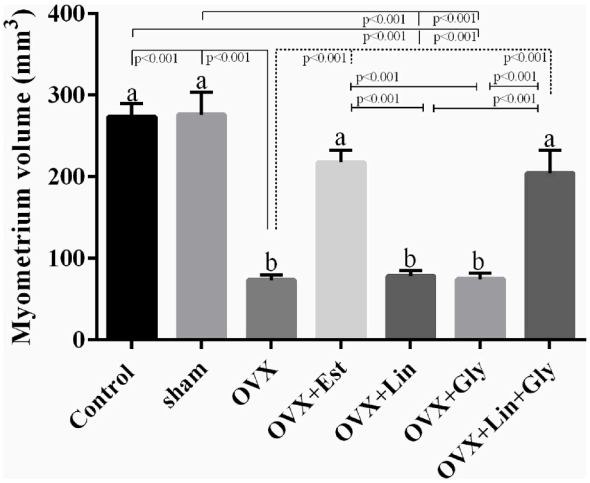

Fig. 7. The average volume of the uterine myometrium layer in the studied groups. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). One-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

3.3. The average volume of the uterine endometrium layer

Data in Fig. 8 shows that ovariectomy (mean = 44.62 mm^3) decreased the endometrial volume significantly concerning the control (mean = 268.9 mm^3) and sham-operated (mean = 296.6 mm^3) groups. The administration of *Linum usitatissimum* oil + licorice (mean = 213.97 mm^3) improved the mentioned parameter in such a way that did not demonstrate any differences compared to the control and sham groups. There was no significant difference in the groups which received *Linum usitatissimum* oil and licorice root extract alone compared to the OVX group.

3.3.1. The average volume of the uterine lumen

As might be expected, ovariectomy (mean = 6.76 mm^3) decreased the lumen volume (Fig. 9) compared to the control (mean = 32.03 mm^3) and sham groups (mean = 37.69 mm^3). *Linum usitatissimum* oil + licorice administration improved the mentioned parameter in such a way that an acceptable increase compared with the OVX group was seen with a mean of 25.19 mm^3 ($F(6,63) = 13.526$, $P < 0.001$ compared with the OVX group).

3.3.2. The average volume of the uterine glands

Similarly, a significant decrease in the average volume of uterine glands was observed in the OVX group (Fig. 10, mean = 4.57 mm^3) compared to the control (mean = 44.68 mm^3) and sham (mean = 43.63 mm^3) groups ($F(6,63) = 10.495$, $P < 0.001$). The average

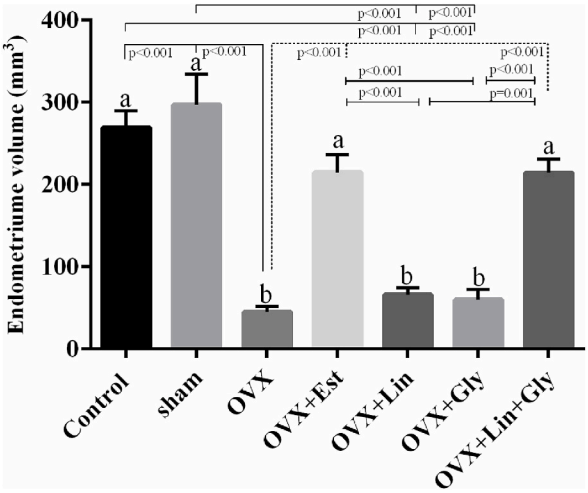

Fig. 8. The average volume of the uterine endometrium layer in the studied groups. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). One-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

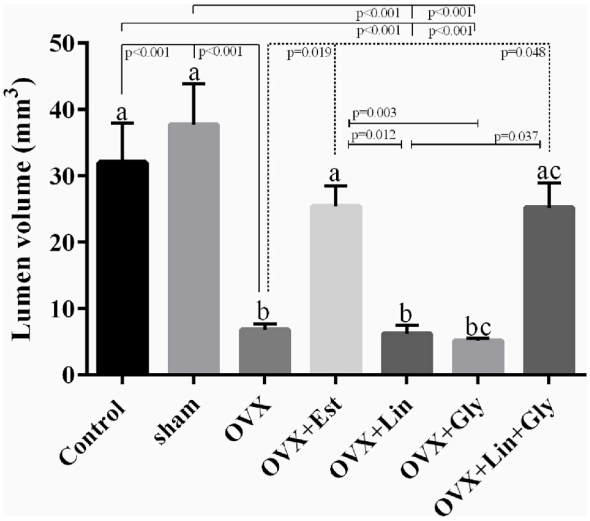

Fig. 9. The average volume of the uterine lumen in the studied groups. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). One-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

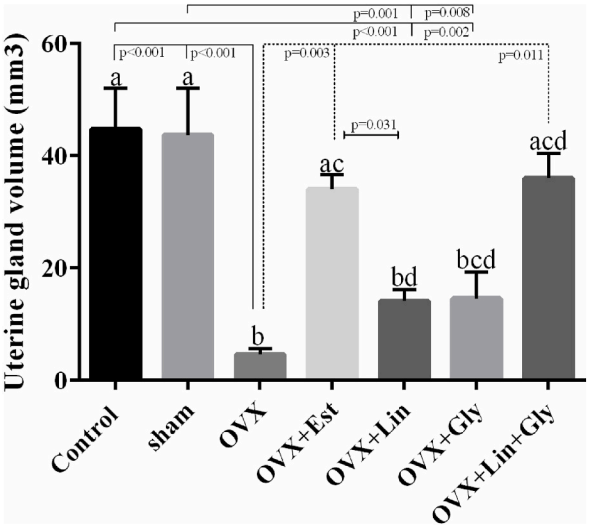

Fig. 10. The average volume of the uterine glands in the studied groups. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). The one-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

volume of uterine glands showed a significant increase compared to the OVX group ($F(6,63) = 10.495$, $P < 0.001$) in the estradiol (mean = 33.96 mm^3) and *Linum usitatissimum* oil + licorice root extract (mean = 35.97 mm^3) receiving groups. There was no significant difference in the groups which received *Linum usitatissimum* oil and licorice root extract alone compared to the OVX group.

3.3.3. *The average thickness of the uterine perimetrium layer*

The OVX tends to decrease the perimetrium thickness although the decline was not significant. The results showed no significant difference between the studied groups (Fig. 11, $P < 0.05$).

3.3.4. *The average thickness of the uterine myometrium layer*

As depicted in Fig. 12, a significant decrease in the average thickness of the uterine myometrium layer was observed in the OVX group compared to the control and sham groups ($F(6,63) = 10.642$, $P < 0.001$). In the OVX groups which received estradiol ($F(6,63) = 10.642$, $P < 0.001$) and *Linum usitatissimum* oil + licorice root extract ($F(6,63) = 10.642$, $P = 0.013$), the mean thickness of the uterine myometrium layer showed a significant increase compared to the OVX group. However, treatment with *Linum usitatissimum* oil and licorice root extract alone was not effective as a flaxseed oil + licorice root extract receiving group.

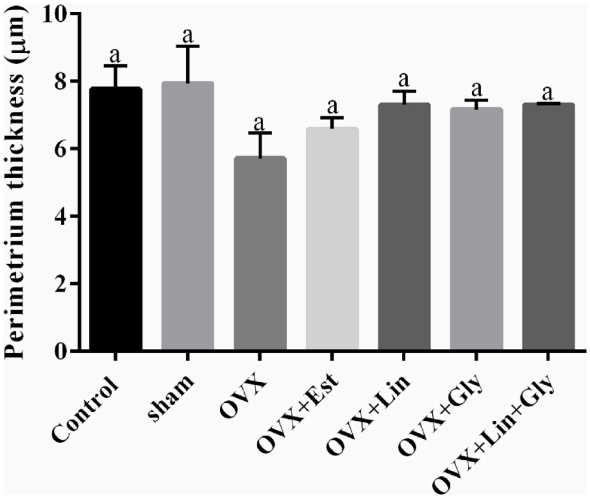

Fig. 11. The average thickness of the uterine perimetrium layer in the studied groups. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). The one-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

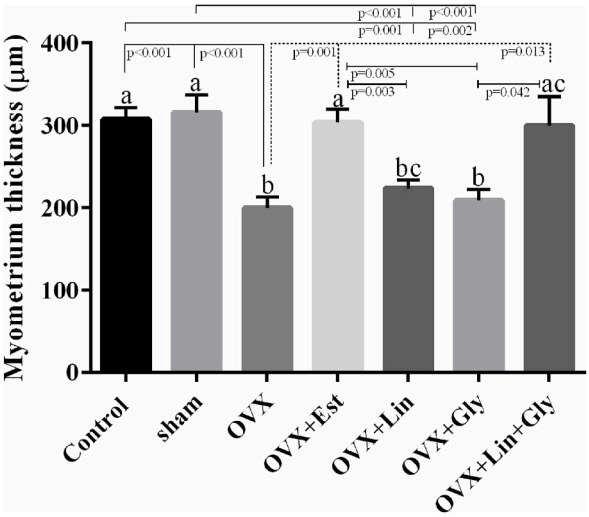

Fig. 12. The average thickness of the uterine myometrium layer in the studied groups. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). The one-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

3.3.5. The average thickness of the uterine endometrium layer

The results of the uterine endometrium thickness are presented in Fig. 13. A significant decrease in the average thickness of the uterine endometrium layer was observed in the OVX group (mean = 223.2 mm^3) compared to the control (mean = 562.9 mm^3) and sham groups (mean = 486.7 mm^3; $F(6,63) = 15.944$, $P < 0.001$). *Linum usitatissimum* oil + licorice root extract (mean = 469.0 mm^3) and estradiol (mean = 477.1 mm^3) groups had a high endometrium thickness, confirming the high efficacy of these treatments. No significant difference was observed in the groups which received *Linum usitatissimum* oil and licorice root extract compared to the OVX group alone.

3.3.6. The average diameter of the uterine tissue vessels

It is well known that uterine tissue vessels are reduced by ovariectomy (Fig. 14). Administration of estradiol, licorice root extract, and *Linum usitatissimum* oil + licorice root extract showed a significant increase in the vessel diameter compared to the OVX group ($F(6,63) = 6.436$, $P < 0.001$, $P = 0.007$, and $P = 0.032$, respectively). In groups that received *Linum usitatissimum* oil alone, the mean diameter of the uterine tissue vessels increased compared to the OVX group, but this increase was not significant ($F(6,63) = 6.436$, $P < 0.05$).

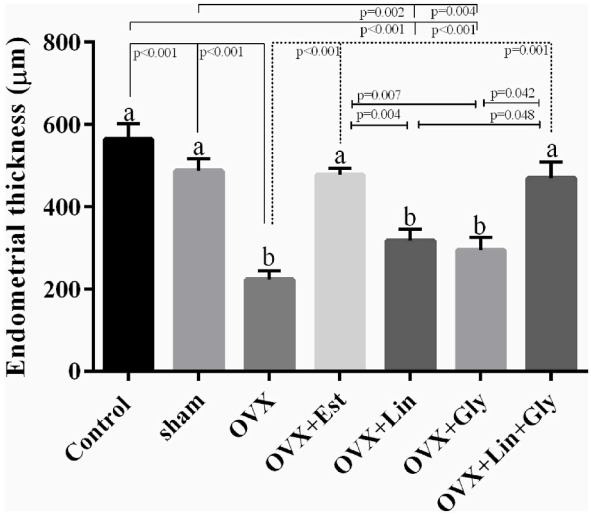

Fig. 13. The average thickness of the uterine endometrium layer in the studied groups. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). The one-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

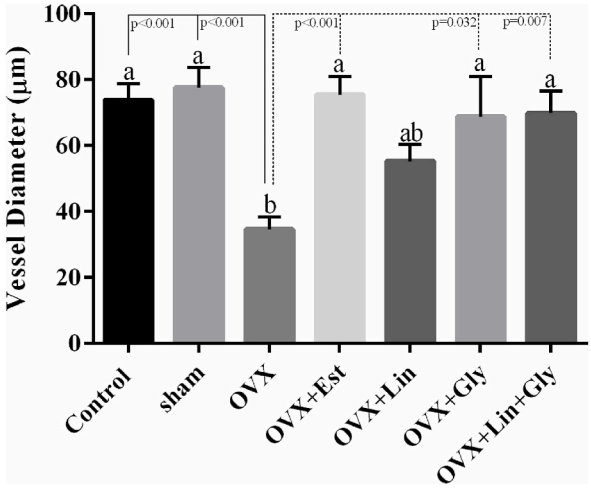

Fig. 14. The average diameter of uterine tissue vessels in the studied groups. The presence of at least one similar letter indicates no significant change ($P < 0.05$) and if there is no similar letter between the groups, it indicates a significant change ($P < 0.05$). The one-way ANOVA method and Tukey post hoc test were used to analyze and compare the results.

4. Discussion

Since no literature data was available on the pharmacological effects of *Linum usitatissimum* oil in combination with licorice, the main goal of this study was to evaluate the therapeutic potential of *Linum usitatissimum* oil and licorice in the OVX rats which generalize the results with menopause and post-ovariectomy problems in women. In this regard, the effects of *Linum usitatissimum* oil and licorice root extract on tissue parameters, hormonal indicators, serum biochemistry, and stereological changes in the uterus of ovariectomized rats were comprehensively investigated.

The uterus in response to changes in the levels of ovarian steroid hormones undergoes periodic alterations of proliferation, differentiation, and shedding. Cellular changes in the uterus during reproductive cycles are controlled by ovarian hormones, especially estrogen, and progesterone [59]. Several studies have shown that the hormones secreted by the ovaries (estrogen and progesterone) affect the uterine tissue, so that estrogen increases the growth of the endometrial layer of the uterus, and progesterone increases the thickness of this layer of tissue [60]. However, the concentrations of progesterone and estradiol in ovariectomized rats were significantly reduced. Decreased circulating estrogen levels are accompanied by increased blood pressure, which contributes to a greater prevalence of hypertension and an increased prevalence of coronary heart disease during the post-menopausal period [61]. During the hormonal lessening, the tissue structure of the uterine wall becomes endometrium atrophy. Removal of the ovaries in female rats

causes genital tissue degeneration, significant structural changes in the tissue, and reduces the volume [62]. All observations of this study were in accordance with the results of previous studies that showed changes in the hormonal cycle and hormonal indicators in ovarian disorders [63,64].

Estrogen can also stimulate bone formation associated with a reduction in hip, spine, and wrist fractures [54,65]. Also, ovariectomy in rats leads to a reduction in calcium concentrations, and bone density causes an increase in intestinal calcium secretion and impairs the calcium balance. Progesterone is involved in the inflammatory response and immune-modulating through decreased cyclooxygenase-2 (COX-2) expression by inhibiting the activity of NF-κB, and negative interactions between the progesterone receptor and NF-κB p65 [66]. Unfortunately, synthetic progestins and estradiol can manifest undesirable side effects because of their interactions with other steroid receptors, such as androgen, estrogen, and glucocorticoid receptors.

Licorice is known as a herbal plant with a unique sweet flavor [67]. More than 20 triterpenoids and 300 flavonoids have been recognized from licorice, confirming the high antioxidant and anti-inflammatory properties of this herbal supplement [26]. The main constituent of licorice is glycyrrhizin, 18β-glycyrrhetinic, phytosterols (sitosterol and stigmasterol), liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, licochalcone A, and glabridin. Licorice can reduce testosterone synthesis and, therefore, can be used to treat women with PCOS [68]. In addition, according to a study on PCOS-induced mice, licorice extract was shown to improve ovarian morphology, oocyte maturation, and embryonic development in a dose-dependent manner (100–150 mg/kg/day for 21 days) compared to the control group. A randomized double-blinded controlled trial on 70 postmenopausal women showed that a cream containing 2% licorice extract prevented vaginal atrophy after eight weeks and led to reduce vaginal pH, vaginal dryness, soreness, itching, and dyspareunia compared with the placebo group [69]. The LH/FSH ratio significantly decreased following treatment with licorice extract with the elimination of PCOS-associated symptoms, including thickening of the theca layer, thinning of the granulosa layer of antral follicles, reduction of the number of the antral follicles, and induction of the number of follicular cysts [70]. The transcription levels of *Cyp11a1* and *Ptgs2* were significantly elevated in licorice extract-treated rats, whereas the mRNA level of *Kitl* did not change in the groups. A major finding of this research was that licorice positively affected IVM and IVF, demonstrating a significantly higher percentage of the oocytes reaching metaphase II and blastocyst stages [71,72]. In detail, glycyrrhizic acid and 18β-glycyrrhetinic acid are considered as inhibitors of inflammation *via* modulation of proinflammatory cytokines and the promotion of immune function [73]. PI3K may play a role in this regulation so that glycyrrhizic acid and 18β-glycyrrhetinic acid can activate the glucocorticoid receptor (GR) signaling by binding to the GR and inhibiting the activity of corticosteroid [74]. Sitosterol and stigmasterol are phytosterols that are chemically similar to animal cholesterol. More specifically, sitosterol is mainly known and used for its cholesterol-like property, and stigmasterol is used as a precursor in the manufacture of synthetic progesterone. It has beeen reported that the mentioned phytosterols play an important physiological role in the regulatory and tissue rebuilding mechanisms related to estrogen effects [75].

In the last years, different authors studied the composition of *Linum usitatissimum* oil, demonstrating that PUFAs such as linoleic acid and α-linolenic acid as the main component of *Linum usitatissimum* oil which regulate prostaglandin synthesis, lessen the severity and minimize the symptoms of chronic inflammatory and inflammatory bowel disease [76,77]. PUFAs consumption reduces the loss of bone weight and strength caused by estrogen deficiency, as would occur in women during post-ovary problems [58]. Interestingly, the synergic effect of estrogen and PUFAs were reported in some studies, showing that PUFAs increase the sensitivity of the receptors toward estrogen and can modulate the binding of hormones. PUFAs are an elemental factor in the actions of estrogen due to altering the expression of receptors on the cells. Another study suggested that PUFAs were shown to down-regulate osteoclastogenic factors and reduce the bone loss in ovariectomized mice [78]. Hence, a combination of PUFAs and estrogen (in our case phytosterol) may be necessary to optimize their benefit and response [79,80]. *Linum usitatissimum* is reported as being effective in the alleviation of the symptoms of premenstrual syndrome. In the study conducted by Mirghafourvand et al. (2016) on 159 women, the effect of flaxseed on the symptoms of premenstrual was examined. The results showed that flaxseed was effective in reducing premenstrual syndrome (50). In another study, the effects of *Linum usitatissimum* oil (2.50 mg/kg body wt) on the OVX rats were shown; ALP, oestrogen, progesterone and calcium levels were improved in the OVX rat. Also, the uterus weight and volume as well as ovarian thickness and volume returned to the normal conditions [10]. Also, *Linum usitatissimum* has been introduced as the first-line treatment for cyclic mastalgia, due to its structural similarity to tamoxifen, and aromatase enzyme inhibitors resulted in a reduction in the severity of breast pain [81]. Improvements in the semen quality were reported during the consumption of *Linum usitatissimum* [82].

In the present study, a significant increase in the activity of the ALP enzyme in the OVX group compared to the control and sham groups was observed. Lack of estrogen leads to bone destruction through a direct effect on the bone cells. In other words, one of the reasons for the increase in the activity of the ALP enzyme in the OVX group is the increase in the activity of the osteoclast cells. Many studies have indicated that HRT was associated with a reduction of hip, spine, and wrist fractures. Noorafshan et al. showed that the number of osteoclast cells in ovariectomized groups increased significantly compared to the control and sham groups [64]. A primary investigation indicated that the licorice and flaxseed oil-enriched diet reduced the amount of ALP enzyme and increased the Ca ions which prevented bone loss. The current study indicated that consumption of *Linum usitatissimum* oil and licorice root extract significantly increased the thickness and volume of the endometrium, myometrium, vessel diameter, lumen volume, and volume of the uterine gland in ovariectomy rats compared to the OVX model. Similarly, Ahmadi et al. indicated that the consumption of licorice root containing phytoestrogen compounds increased the diameter of the uterus [83]. The protective effect of *Linum usitatissimum* oil in the ovariectomized rat was also reported by Boulbaroud et al. [84]. In another study, it was seen that the consumption of *Linum usitatissimum* reduced the tumor growth in ovariectomized mice, and long-term consumption of phytoestrogen-rich diet stimulated the production of estrogen [85]. Studies have evaluated the biological mechanism of PUFA increasing leptin and IGF-1 levels and improving the mechanical properties of the bone [86]. Studies on licorice about the bone health in postmenopausal women and prostate health in men have shown promising results. Simmons et al. showed the antagonistic properties of the licorice extract on

estrogen receptors in laboratory [87]. Licorice is also used to reduce the effects of menopause, according to traditional medicine sources [83]. Frattaruolo et al. showed that the extract of licorice, *in vitro*, had considerable antioxidant properties due to flavonoids and other phenolic compounds. Interestingly, licorice works as modulation of nuclear factor kappa B/mitogen-activated protein kinases (NF-kB/MAPK) which markedly decreased pro-inflammatory cytokines and cyclooxygenase 2/inducible nitric oxide synthase (COX-2/iNOS) expression levels [88]. Dangguijagyagsan (100 mg/kg/day for 5 week), as a traditional herbal prescription, has beneficial effects in terms of preventing cardiovascular disease in a menopausal woman *via* reducing the serum lipid levels and improving the blood flow by inhibiting platelet aggregation and thrombus formation [89]. Similarly, ethanol extract from the root of *Morinda officinalis* (2.0 g/kg for 12 weeks) due to the presence of high amounts of anthraquinones and polysaccharides improved the levels of phosphorus (P), calcium (Ca) and OPG, and decreased the levels of DPD/Cr, TRAP, ACTH and corticosterone [90]. Also, 1 g/kg of methanolic extract of *Cuminum cyminum* fruits for 10 weeks significantly reduced the urinary calcium excretion and significantly increased the calcium content and mechanical strength of the bones in comparison to OVX control. It showed greater bone and ash densities and improved the microarchitecture of the bones in SEM analysis [91]. Assessments on different doses of ethanol extract *of Cissus quadrangularis*, 500 and 750 mg/kg for 3 months on OVX rat induced antiosteoporotic effect and improved the biochemical parameters [92]. However, in another rearch article, it was reported that consumption of 500 mg/kg body weight daily for 8 weeks of anthocyanin-rich bilberry extract did not improve the general condition and did not prevent the OVX-induced bone loss under experimental conditions [93].

The main findings of this study were as follows: (i) combined therapy with licorice and *Linum usitatissimum* oil restored endothelial dysfunction caused by estrogen deficiency in rats; (ii) improved the stereological parameters (thickness and volume of the endometrium, perimeter, myometrium, vessel diameter, lumen volume, uterine gland volume), and (iii) normalizes hormonal and serum indices in OVX model. Concerning the beneficial properties of licorice root hydroalcoholic extract and *Linum usitatissimum* oil and minor side effects in OVX animal studies, such natural medications can be evaluated as therapeutic candidates in menopausal and postmenopausal women under the control condition to reduce the complications of the chemicals.

The main limitation of the current study is that the exact mechanisms that explain the mechanism of licorice root hydroalcoholic extract combined with *Linum usitatissimum* oil in OVX rats are lacking in correctly interpreting the results of animal models into clinical practice. In the current study, 2.0 mg/kg body wt licorice root hydroalcoholic extract and 2.0 mg/kg body wt *Linum usitatissimum* oil were used. Assessments on different dosages of the mentioned plants might affect the effectiveness of treatments. In the next step, *in vivo* toxicological evaluations should be considered and in the future, some relevant clinical trial studies might be conducted to ensure that medical intervention and treatment are safe and effective.

5. Conclusion

In this study, for the first time, the effect of *Linum usitatissimum* oil and licorice extract, simultaneously, on the ovariectomized rats was investigated. The results showed that ovariectomy caused stereological and histological changes in the uterus and hormonal and serum indices. This study showed that a combination of this oil and extract can be used as a complementary and inexpensive treatment for OVX. It seems that such multi-therapeutic approach can reduce the side effects during menopause and post-ovariectomy problems in women suffering from reproductive diseases and the chemotherapy process. The findings of this study provided new insights into the effects of *Linum usitatissimum* oil and licorice extract on human health as HRT.

Author contribution statement

Nader Tanideh: Conceived and designed the experiments; Performed the experiments. Fatemeh Daneshmandb: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Marzieh Karimimaneshb: Performed the experiments; Contributed reagents, materials, analysis tools or data. Javad Mottaghipishehc; Romina Tanideha: Contributed reagents, materials, analysis tools or data; Wrote the paper. Farhad Koohpeymad: Analyzed and interpreted the data; Wrote the paper. Omid Koohi-Hosseinabadi: Performed the experiments. Cambyz Irajie; Aida Iraji: Conceived and designed the experiments; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of competing interest

The authors of this manuscript declare no conflict of interests.

Availability of data and materials

Adequate and clear descriptions of the applied materials and methods are provided in the materials and method section of the manuscript.

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