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Histomorphometrical changes of rat uterine and ovarian tissues fed chloroform fraction of dill (*Anethum graveolens* L.) extracts

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Abstract

The effects of dill (*Anethum graveolens* L.) seed extracts have studied previously. Study of different fractions of an extract helps us to understand which one of its components act on target organs. In this study, 42 female rats were divided into 6 groups of control, sham, and low (0.5, 0.045 g/kg) and high (5, 0.45 g/kg) doses of chloroform fraction of dill seed aqueous and ethanol extracts. During 10 days, rats received 1ml of extracts, then uterus and ovary were removed and their histological sections were prepared, stained by H&E and Masson's trichrome and then morphometry was performed. Glycoconjugates of cell surface of reproductive organs were stained by lectins DBA, PNA, UEA, ConA and SBA) and their intensity of reactions were measured by Imag-Java software. Morphological studies showed that uterine diameter was decreased in low dose of aqueous extract and high dose of ethanol extract. Myometrial diameter was decreased by high dose of ethanol extract. Histological studies using lectin ConA revealed lower intensity of reactions of oocyte cell membrane, endometrial and myometrial layers in high dose of ethanol extract treated group compared to the control group. It was concluded that chloroform fraction of dill extracts can affect female reproductive organs.

Keywords: Chloroform fraction, Dill extracts, Histomorphometry, Lectin histochemistry, Ovary, Uterus

1. Introduction

Pregnancy and childbirth is very important in human culture and it is main causes of women's physical and mental development. However, population growth and its control is one of the important problems of many societies. The side effects of various contraceptive methods are reasons for research of new contraception methods. The uses of medicinal plants have long history and nowadays many developing and developed countries encouraged traditional medicine for treatment of some diseases. In Iranian traditional medicine *Anethum graveolens* L. (dill), belong to umbeliferae family reduces menstrual irregularity and pain. Also it is used as food flavor and as aroma in cosmetics products (1, 2). Our previous studies showed that dill seed aqueous and ethanol extracts increased plasma progesterone concentration and the duration of diestrus phase of the estrous cycle (3). These results suggested that dill can be used as either a regulatory or an anti-fertility agent. The ultra-structural study of corpus luteum revealed smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER) and mitochondria increased in corpus luteum granulosa cells, which indicated more steroidal hormone synthesis (4).

Since study of different fractions of herbal medicine extracts helps us to understand which components of extract act on target organs, our previous study showed that chloroform fraction of dill seed aqueous and ethanol extracts reduced estradiol concentration and increases the duration of mating to fertilization (5). It means that extract could delay sperm and oocyte interaction and fertilization. So we concluded that it caused by different mechanism such as histological changes or glycoconjugate alteration of uterine and ovarian structure. Therefore in the present study we examined the lectin histochemistry and histomorphometrical changes of endometrium, myometrium and ovarian

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follicles in female rats treated with chloroform fraction of dill seed aqueous and ethanol extracts.

2. Materials and Methods

2.1. Extracts and fraction preparation

Dill seeds were purchased from a commercial source in Shiraz (Fars province, southern of Iran). The identity of the seeds was confirmed by a botanist in Biology Department, Shiraz University, Shiraz, Iran and a voucher specimen (1015) was kept at the Herbarium of the this department. Seeds were then powdered and 100 g of the powder and 300 ml of 80% ethanol (for ethanol extract), as well as 100 g of the powder and 300 ml of distilled water (for aqueous extract), were percolated for 24 hours. Subsequently, the mixtures were filtered and concentrated under a reduced pressure using a rotary evaporator. The yields (w/w) of the aqueous and ethanol extracts were 8.2% (g/g) and 4.5% (g/g) solid residue, respectively. In the subsequent course, 20 g of the aqueous extract and 40 g of the ethanol extract were independently rinsed three times with chloroform at the room temperature. The oily liquids were evaporated to dryness under vacuum conditions. The yields (w/w) of chloroform fraction of the aqueous extract were 0.5% (g/g), while the yields (w/w) of chloroform fraction of ethanol extract were 0.25% (g/g) solid residue.

2.2. Animals grouping

Adult Wistar female rats (10–12 weeks old, 150–200 g) were randomly drawn from the stock colony of the animal house of the Razi Institute of Shiraz, and were housed individually in polypropylene cages under standard housing conditions (controlled atmosphere with 12:12-hour light/dark cycles and an ambient temperature of 22-24 °C). The rats were acclimatized for two week prior to the start of the experiment. Rats were maintained on commercial pellet diet (Javaneh Khorasan Company, Mashed, Iran) and had free access to food and tap water. All procedures with animals were conducted strictly in accordance with approved guidelines by the National Institute of Health (6) followed at all times, and maximum care was taken to minimize animal suffering, in addition, the number of rats used was kept at a minimum. Animals were weighed before, middle and after the experiments. Vaginal smear were examined daily and finally rats with normal estrous cycles were selected. Female rats with regular estrous cycles were randomly divided into six groups of seven each. Control group (CON) received 1 ml distilled water, sham group (SHAM) received 1 ml DMSO (Dimethyl sulfoxide) as a solvent of the chloroform fraction and the experimental groups that received either a low (0.5 g/kg) or a high dose (5 g/kg) of chloroform fractions of the aqueous extract (LDAE and HDAE respectively), and low (0.045 g/kg) or a high dose (0.45 g/kg) of chloroform fractions of the ethanol extract (LDEE and HDEE respectively). All experimental groups were treated fraction per kg body weight in 1 ml of DMSO daily for 10 days (2 regular estrous cycles).

2.3. Histological study

The left ovary and uterus of each rats were cut and fixed in 10% buffered formalin, dehydrated through ascending grades of ethanol (70%, 90%, and 95%, v/v), cleaned in xylene, and embedded in paraffin wax (melting point 56 ℃). Serial sections were cut using rotary microtome (Zeiss, Germany) at 7 microns thickness. The sections were stained with haematoxylin-eosin and Masson's trichrome according to Bancroft and Stevens (7). Finally, photographs of the prepared slides were taken by optical microscope and morphometric analysis was performed by ocular micrometer (Zeiss, Germany). In the transverse section of the uterus, diameters of endometrium, myometrium, length of uterine gland and its epithelial were measured. Longitudinal and transverse diameters of ovary, secondary follicles and corpus luteum diameters, granulosa cells cytoplasm and nucleolus diameters were measured. Histomorphometric studies were examined in 3 microscopic slides of each animal.

2.4. Lectin histochemistry

Deparaffinized and dehydrated sections were incubated in PBS containing 0·1mM-$CaCl_2$, $MgCl_2$ and $MnCl_2$. The endogenous peroxidase was blocked by incubating the sections in 1% H_2O_2 in methanol for 10–15 min. The specimens were then incubated with peroxidase-conjugated lectins (Sigma, USA) for 2 h at room temperature. The lectins of *Ulex europeaeus* agglutinin (UEA), Concanavalin A (Con A), *Dolichos biflorus* agglutinin (DBA), Peanut agglutinin (PNA), Soybean agglutinin (SBA) which bind specifically to α -L-fucose, α –mannose, α-N acetyl galactose amine, N acetyl galactose amine and α & β-N acetyl galactose amine respectively, were used at a final concentration of 10mg/ml. After washing, the binding sites were visualized by incubating the sections in 0·03% diaminobenzidine containing 200 ml H_2O_2 in PBS for 10 min. Then, the sections were counterstained with alcian blue (0·5 %). Photographs were taken with a digital camera and the intensity of the reaction to each lectin was assessed with Image-Java software.

2.5. Data analysis

The data were expressed as one-way ANOVA, followed by the Tukey and Scheffe tests. Statistical analysis were done with SPSS v. 11.5 software and $p<0.05$ between the experimental groups at each point were considered statistically significant.

3. Results

Histological study of the uterus after routine staining (Fig. 1) and Masson's trichrome staining (Fig. 3) revealed that the tissue structure of endometrium, myometrium and perimetrium did not have any pathological changes in the experimental groups receiving low and high doses of chloroform fractions of dill seed aqueous and ethanol extracts compared to the control group. The ovarian follicles including primordial, primary, secondary and mature follicles, corpus luteum and tissue structure of ovarian medulla revealed no changes in the experimental groups compared to the control group (Fig. 2 and 3).

The results showed that the total diameter of the uterus in the LDAE treated group, uterine wall thickness and endometrial and myometrial diameters of HDEE treated group decreased significantly compared to the control. The length of uterine glands in the group receiving the high dose of aqueous extract showed a significant decrease compared to the control group. Morphometrical analysis of the epithelial height in the experimental groups showed no significant differences compared to the control group (Table 1).

Ovarian morphometrical analysis revealed that the longitudinal diameter of the ovary in the groups receiving chloroform fraction compared with the control group have no significant difference. Transverse diameter of the ovary in groups receiving low and high doses of aqueous and ethanol extracts of chloroform fraction were significantly reduced compared to the control group (Table 2).

Daily dietary supplementation with chloroform fraction of dill seed aqueous and ethanol extracts changed the proteoglycan content of the oocyte. Also oocyte cell membrane, endometrium and myometrium layers of the uterus reacted less intensely to ConA in HDEE-treated group compared with the control group (Fig. 4 and 7). Intensity reaction of oocyte and granulose cells glycoconjugates after staining with ConA lectin in rats that treated with aqueous and ethanol extracts of chloroform fractions of dill seed decreased in experimental groups compared to the control group. There were no significant difference in the intensity of the reactions to UEA, DBA, PNA and SBA in the granulosa cells, oocyte, endometrium, myometrium and ovarian connective tissue after extracts treatment (Fig. 4-7).

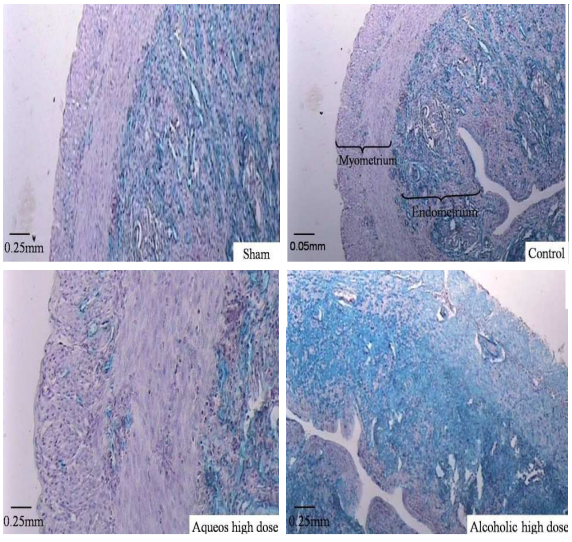

Figure 1- Transverse sections of uterus in rats treated with aqueous and ethanol extracts of chloroform fractions of dill seed. Masson's tri-chrome staining.

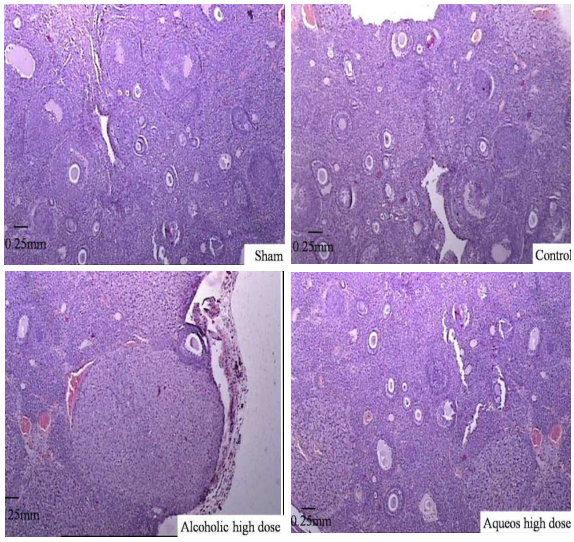

Figure 2- Transverse sections of ovary in rats treated with aqueous and ethanol extracts of chloroform fractions of dill seed. Haematoxylin-eosin staining.

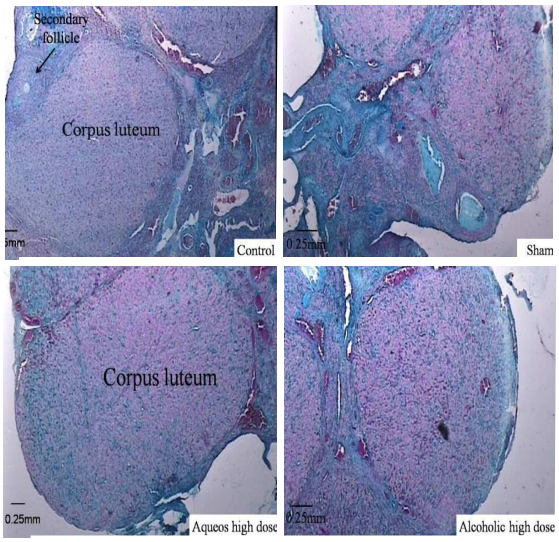

Figure 3- Transverse sections of ovary in rats treated with aqueous and ethanol extracts of chloroform fractions of dill seed. Masson's tri-chrome staining. Scale bar=0.25µm.

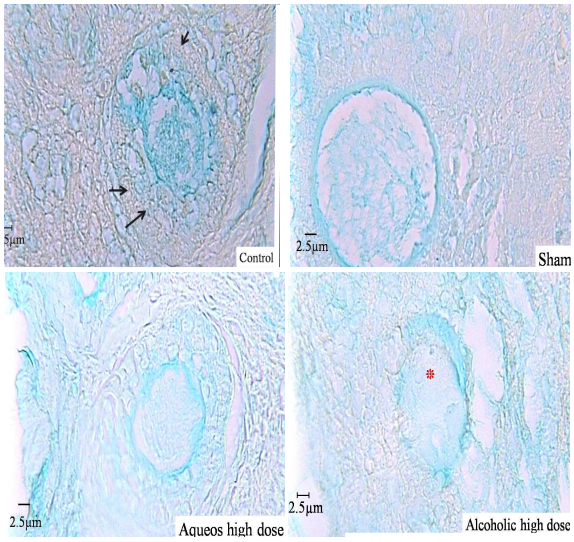

Figure 4- Intensity reaction of oocyte and granulose cells glycoconjugates after staining with ConA lectin in rats that treated with aqueous and ethanol extracts of chloroform fractions of dill seed.

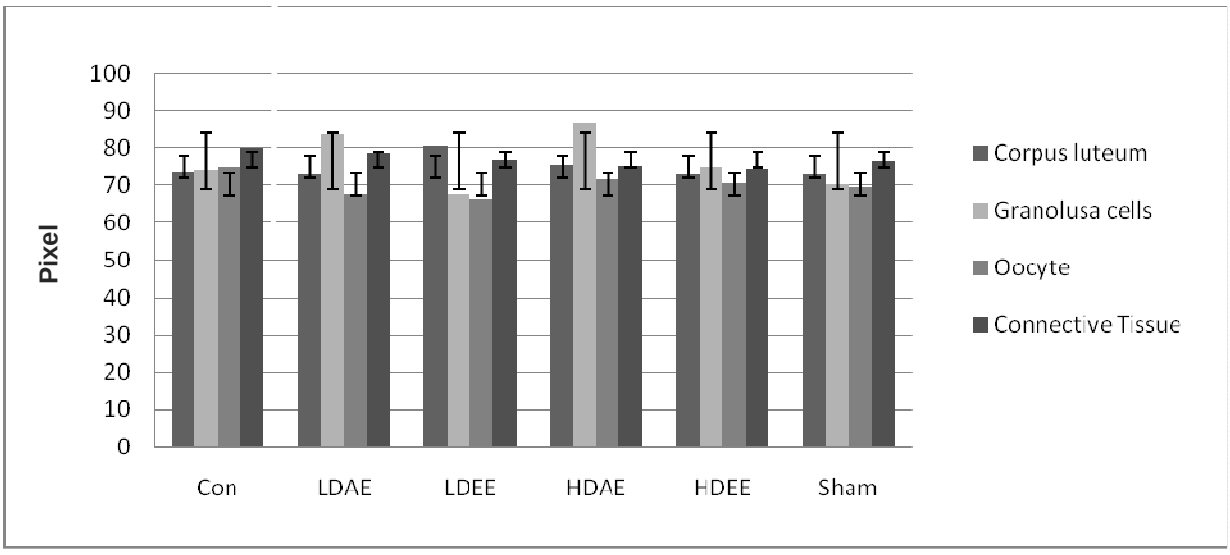

Figure 5- Intensity reaction of ovarian glycoconjugates after staining with PNA lectin in rats that treated with aqueous and ethanol extracts of chloroform fractions of dill seed. CON: control; LDAE: low dose of aqueous extract; LDEE: low dose of ethanol extract; HDAE: high dose of aqueous extract; HDEE: high dose of ethanol extract

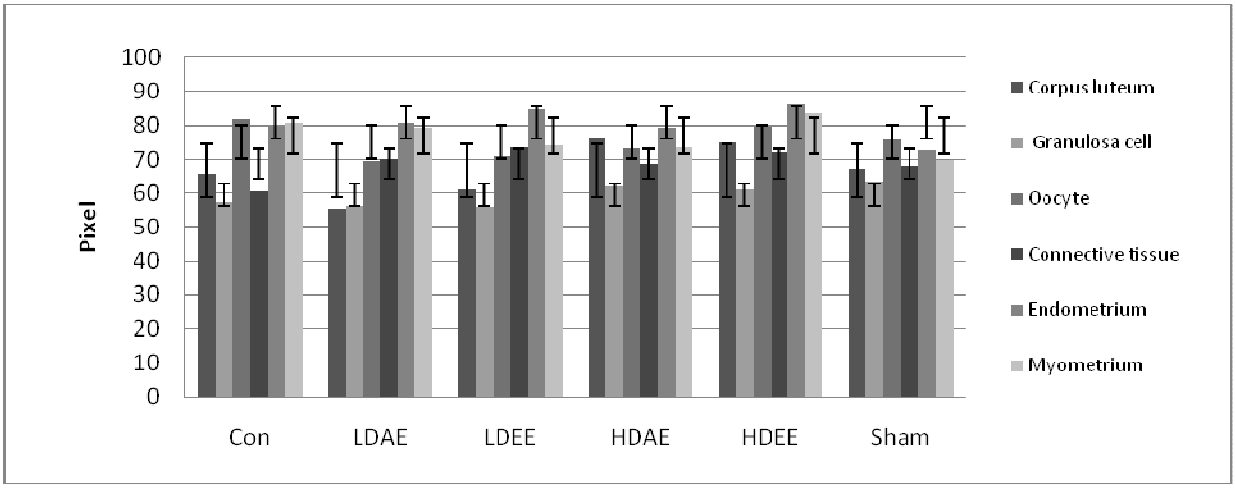

Figure 6- Intensity reaction of uterine and ovarian glycoconjugates after staining with DBA lectin in rats that treated with aqueous and ethanol extracts of chloroform fractions of dill seed. CON: control; LDAE: low dose of aqueous extract; LDEE: low dose of ethanol extract; HDAE: high dose of aqueous extract; HDEE: high dose of ethanol extract

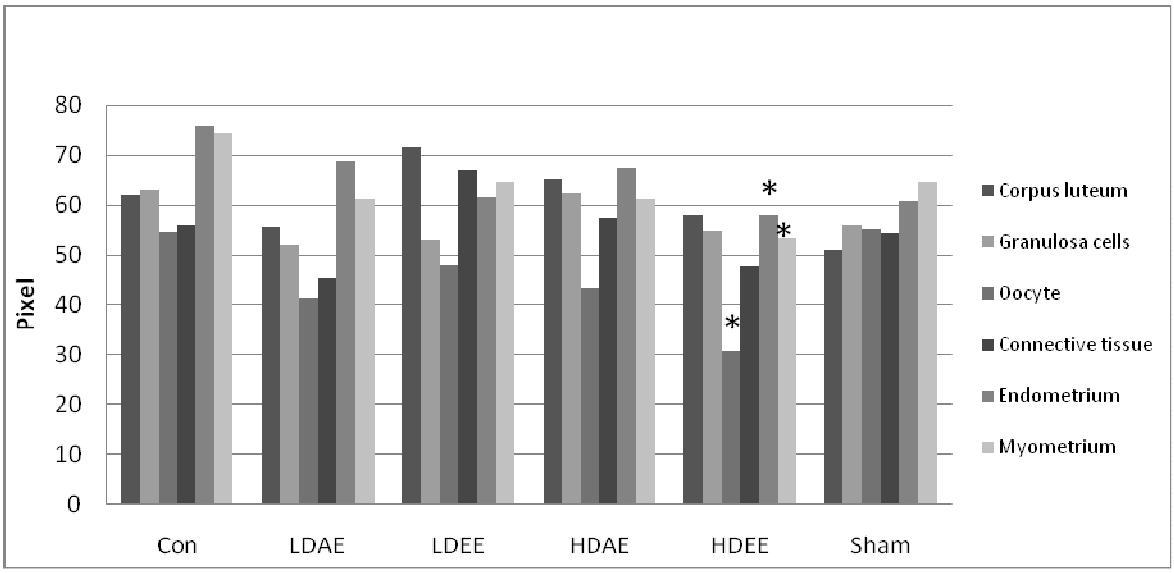

Figure 7- Intensity reaction of uterine and ovarian glycoconjugates after staining with ConA lectin in rats that treated with aqueous and ethanol extracts of chloroform fractions of dill seed. CON: control; LDAE: low dose of aqueous extract; LDEE: low dose of ethanol extract; HDAE: high dose of aqueous extract; HDEE: high dose of ethanol extract.

Table 1. The uterine morphometry (mm) in rats treated with chloroform fractions of aqueous and ethanol extracts of dill seeds. Values expressed as means ± S.D.

Groups	Total diameter	Uterine wall	Endometrial diameter	Myometrial diameter	Length of glands	Epithelial length
CON	2.67±0.16	1.29±0.28	0.91±0.28	0.38±0.06	0.92±0.34	0.11±0.02
LDAE	2.01±0.28*	1.17±0.16	0.74±0.19	0.42±0.10	0.90±0.53	0.12±0.02
LDEE	2.44±0.41	1.32±0.13	0.91±0.12	0.40±0.04	0.89±0.31	0.10±0.03
HDAE	2.24±0.26	1.28±0.19	0.79±0.10	0.40±0.11	0.40±0.06*	0.11±0.01
HDEE	2.31±0.73	0.77±0.15*	0.53±0.13*	0.27±0.02*	0.82±0.05	0.11±0.05
SHAM	2.38±0.44	1.18±0.28	0.80±0.23	0.39±0.10	0.72±0.37	0.11±0.03

* Show significant differences with the control group (P<0.05)
CON: control; LDAE: low dose of aqueous extract; LDEE: low dose of ethanol extract; HDAE: high dose of aqueous extract; HDEE: high dose of ethanol extract

Table 2. The ovarian morphometry (mm) in rats treated with chloroform fractions of aqueous and ethanol extracts of dill seeds. Values expressed as means ± S.D.

Groups	Ovarian diameter		Corpus luteum	Granulosa cells diameter		Secondary follicle diameter	
	Longitudinal	Transverse		Cell	Nucleus	Longitudinal	Transverse
CON	3.59±0.53	3.05±0.10	2.05±0.21	10.10±0.21	7.90±0.18	0.23±0.08	0.19±0.06
LDAE	2.78±0.30	2.13±0.08*	2.02±0.31	7.30±0.04	6.90±0.16	0.20±0.12	0.15±0.10
LDEE	2.94±0.18	1.68±0.08*	2.50±0.69	8.00±0.15	7.40±0.20	0.35±0.06	0.24±0.04
HDAE	2.90±0.20	2.06±0.03*	2.72±0.06	7.00±0.10	6.50±0.24	0.24±0.01	0.23±0.03
HDEE	3.29±0.24	2.26±0.11	2.98±0.35	9.10±0.13	8.10±0.04	0.25±0.04	0.23±0.03
SHAM	3.90±0.38	3.17±0.49	3.03±0.53	10.60±0.15	9.80±0.15	0.22±0.00	0.21±0.05

* show significant differences with the control group (P<0.05).
CON: control; LDAE: low dose of aqueous extract; LDEE: low dose of ethanol extract; HDAE: high dose of aqueous extract; HDEE: high dose of ethanol extract

4. Discussion

Light microscopic observations revealed no pathological changes such as necrosis, abnormal cells or congestion in transverse sections of uterine and ovary after treated with extracts. Therefore it was concluded that chloroform fraction of dill seed aqueous and ethanol extracts is safe and has not side effects on tissue structures.

Morphometrical analysis showed that transverse diameter of the ovary and uterine wall thickness include myometrial and endometrial diameters decreased significantly especially in high dose administrated groups.

Dill consisted of some flavonoids like kaempferol, myristicin and vicenin. Kaempferol and vicenin have phytoestrogen properties (8, 9). Phytoestrogens are nonsteroidal component of plants that similar to natural estrogens such as 17β-estradiol, they attached to alpha and beta estrogen receptors and induce biological effects (10). Monsefi et al reported that dill seed extract caused the survival of corpus luteum and dilated its granulosa lutein cells' SER that causes high secretion of progesterone. It caused to prolong diestrus phase of estrous cycle and inhibit ovulation in the next cycle.

These results are similar to steroidal contraceptive pills performance and it may relate to phytoestrogenic components of dill extracts (3, 4). Phytoestrogens inhibit tyrosine kinase activity and prevent myoblast proliferation and myotube protein synthesis. They affect *HoxA10* gene expression that is effective on reproductive system development. Also phytoestrogens are able to decrease endometrial thickness (11). Therefore it may be suggested that lower thickness of uterus and ovary are caused by some component of chloroform fraction of dill seed aqueous and ethanol extracts. Our results revealed that the distribution of α-mannose terminal sugar of

oocyte cell membrane, endometrial epithelial cells and myometrial muscle cells glycoconjugates decreased in HDEE treated groups after staining with Con A. Glycoconjugates played important roles in cell diagnosis, cell differentiation, cell migration, apoptosis, cell cycle control and capacitation. Apical surface of endometrial epithelial cells of monkey reacted with Con A one day before implantation (12). The shape and function of intercellular adhesion molecules (ICAMs) regulated by mannose (13).

Modifications in the oocyte glycoconjugates are important for gamete recognition and fertilization in mammals. Alteration in endometrial glycoconjugates could affect implantation. These are very important points that could cause infertility. Female's rats that treated with chloroform fractions of dill seed aqueous and ethanol extracts fertilized 7-10 days (1 or 2 estrous cycles) later than to the control rats (5).

There were 9-11 days delayed times when the male rats that fed with dill seed aqueous and ethanol extracts mated with female rats (5). Therefore it may be conclude that chloroform fractions of *Anethum graveolens* (dill) seed aqueous and ethanol extracts affect fertility potential in rats as an animal model.

Conclusion

Chloroform fractions of *Anethum graveolens* (dill) seed aqueous and ethanol extracts did not change histological structure of uterine and ovaries but it decreased ovary, endometrial and myometrial thickness and altered intensity of reactions of α-mannose terminal sugar glycoconjugates of oocytes, endometrium and myometrium.

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