Original Article

# Effect of hydroalcoholic extract of pumpkin seeds on start of oogenesis and change of hormone-pituitary-ovarian axis in immature rats

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## Abstract

**Introduction:**
Pumpkin seed has been used in traditional medicine as a drug for improving sexual function and vigor. We did not find sufficient scientific evidence to support it. The present study was designed to evaluate the effect of hydroalcoholic extract of pumpkin seeds on hormone-pituitary-ovarian axis and oogenesis in immature rats.

**Materials and Methods:**
In this experimental study, we used 30 immature female Wistar rats (weight range 100 to 80 *g* and 35-30 days old) were randomly selected and divided into five groups of six, three experimental groups, control and sham groups. The experimental group was injected 200,100,50 *mg/ kg* dose of pumpkin seed extract intraperitoneally for 21 consecutive days. One day after the last injection, blood was taken from the rats to investigate the sex hormones, and then their ovaries were removed for histological studies.

**Results:**
The results showed a significant increase in the mean serum FSH, estrogen and progesterone in the experimental groups compared to the sham and control ($P<0.05$). Secondary follicles in the experimental group compared to the sham and control groups showed a significant increase ($P<0.05$). Body weight and weight of the right and left ovaries in the experimental group increased ($P<0.05$). Amongst the other parameters, no significant differences were observed in all groups.

**Conclusions:**
Injection of hydroalcoholic extract of pumpkin seeds could significantly be effective on oogenesis and concentration of FSH, estrogen and progesterone hormones.

**Keywords:** Plant Extract, Luteinizing Hormone, Follicle Stimulating Hormone, Oogenesis, Estrogen, Progesterone

## Introduction

Maturity is a period in life associated with specific sexual, physical and cerebral symptoms and includes certain physical changes along with changes in the hypothalamic-pituitary-gonadal axis (1). In recent years, the side effects of chemical drugs, certain drug allergies in people and the increase in disease resistance to chemical drugs have switched the focus of

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researchers to the use of herbal products in various fields of medicine (2). For example, hormone replacement therapy is used to treat failure in maturity. In fact, hormone replacement therapy refers to a combination of estrogen and sometimes progesterone associated with the risk of hyperplasia, endometrial cancer, breast cancer and thromboembolism (3-4).

Nature is a rich source of medicinal compounds that are partly hidden in plants. For thousands of years, plants have been widely used as traditional medications. Recently, there has been a great shift of attention in developing countries to the use of herbal medicines in the treatment of various diseases and the use of natural antioxidants in the food industry due to the side effects of synthetic drugs; studies are rapidly being conducted on the extraction of biologically active compounds from plants (5).

Pumpkin and its seeds are considered a popular traditional native American food with unique nutritional and health values. Pumpkin seed is sometimes used in European cuisine –mainly in the southern parts, Austria, Hungary and Slovenia. Today, the United States, Mexico, India and China are among the largest commercial pumpkin growers (6).

Pumpkin is the genus of Cucurbita from the Cucurbitaceae family (7-8, 9-10) with dark green flat seeds surrounded by a white shell (11). The present study was conducted on pumpkins of the C. Maxima species. These pumpkins are orange and have more benefits than other species of pumpkins (12). The genetic modification of the C. Maxima pumpkin and its agricultural diversification originates from Spain, South America (13). In addition to proteins, vitamins and antioxidants, pumpkin seed is also rich in carotenoids and tocopherol (14).

In traditional medicine, eating the pumpkin seed kernel relieves and heals dysuria, peptic ulcer and bloody sputum and coughing (11). Raw pumpkin seeds help treat intestinal parasite worms and are also used to treat prostate cancer and hypertrophy. Pumpkin seed oil is used for healing fever and nausea. Pumpkin and its seeds are highly effective in preventing lung cancer (15). This seed is considered a useful source of nutrients for humans (16).

A study conducted in 2010 by Abd El-Ghany et al. showed that the daily consumption of pumpkin seed and a diet high in zinc help improve sexual health (17). A study conducted in 2006 by Gossell et al. found that the consumption of pumpkin seeds rich in phytoestrogen, which is a precursor for the synthesis of estradiol, increases estradiol levels (18). A 2010 review study by Shamloul reported that despite the increasing tendency to use herbal medicines for the improvement of sexual activity and erectile dysfunction, there is not enough scientific evidence to confirm their effects (19). This study was thus conducted to investigate the effect of hydroalcoholic extract of pumpkin seeds on oogenesis and pituitary-ovarian axis in immature rats.

## Materials and methods

The Soxhlet method was used for squeezing the hydroalcoholic extract (80%) of the pumpkin seed. To this end, 100 grams of dried pumpkin seeds were milled to powder, mixed with 500 ml of 80% ethanol and then kept in the laboratory in a percolator for three days. After three days, the extract was collected drop by drop through the valve at the bottom of the device and 80% ethanol was added until the extracted liquid became colorless which indicated that there was no longer any extract remaining. The next stage comprised of passing the mixture through filter paper to make it clear. The extract was then evaporated in a rotary evaporator at 40°C temperature to increase its concentration. A desiccator was then used to expose the extract to strong vacuum for 24 hours so as to dry it up. The dried extract was then weighed. This process yielded 16 grams of dried extract from 100 grams of pumpkin seed powder.

Concentration of the extract is thus 16% (20).

In order to determine the appropriate doses for pumpkin seed extract in this study, different concentrations of pumpkin extract were randomly injected into five different groups of six rats each. The group in which half the rats were alive and half were dead was considered the LD50 concentration group. Next, according to the LD50 concentration obtained (400 mg/kg), the highest, median and lowest concentrations were determined. For the maximum dose, 200 mg (half of the LD50 value), and for the mean and minimum doses, 100 and 50 mg/kg of pumpkin seed extract was dissolved in 1 ml distilled water.

This experimental study was conducted on 30 immature female Wistar rats aged 3 weeks and weighing from 80 to 100 g.

The rats were procured from the animal house of the Islamic Azad University of Jahrom. During the study, animals were fed ad lib and were provided with the 12 hours darkness 12 hours light cycle and were housed for two weeks before the beginning of the experiment for adaptability purposes. This study has observed all the ethical principles of working with laboratory animals.

Based on the animals' weight, the required amounts of extract were drawn using insulin syringes at 100, 50 and 200 mg/kg doses. The rats were randomly divided into five groups of six rats each, i.e. the experimental groups 1, 2 and 3, the control group and the sham control group. The experimental groups were given 0.1 ml of the 50, 100 and 200 mg/kg of body weight solution of the pumpkin seed extract and the sham control group was given 0.1 ml of intraperitoneal normal saline solution for 21 consecutive days. The control group did not receive any medication.

One day after the termination of the 21-day period, all the rats were anesthetized and blood sample was taken from their hearts. Blood serum was separated using a Pasteur pipette and the centrifuge method and was stored at -20°C.

The hormone levels were measured using the ELISA method and its specific kit for measuring estrogen and progesterone hormones (DRG Co., Germany) and the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) kits (Pishtaz Teb Co., Iran).

Once blood sample was collected, an incision was made on the rats' abdomen and their ovaries were removed from the surrounding fat tissues and the fallopian tube using scalpel and forceps. Once weighed on a digital scale, the ovaries were washed with normal saline solution and were each kept in formalin 3% for 14 days. Finally, in the histology laboratory of Shiraz MRI Hospital, a minimum of five slides of the ovaries were prepared for further examination. In the slides prepared from the different parts of the ovary, vascular congestion, vacuolization of ovarian tissue cells, follicles atresia, the mean of primordial, primary, secondary, Graafian and atretic follicles and the corpus luteum were examined using an optical microscope with 400-fold magnification and the number of follicles were counted. The mean number of follicles was determined for each group and then compared against other groups.

These examinations showed that conditions for using the analysis of variance (ANOVA) test are met. Data obtained from the hormonal measurement and the ovary weights were analyzed using the statistical software SPSS 17, one-way ANOVA and Tukey test (pairwise comparison). $P \leq 0.05$ was defined as the significance level.

## Results

Results of this study are all presented in Table 1 and Figures 1, 2, 3 and 4.

Table 1 Comparison of values of the variables in different groups

| | Control | Sham Control | Experiment 1 | Experiment 2 | Experiment 3 |
|---|---|---|---|---|---|
| Animal weight (g) | 116.2±1.496 | 116.1±1.48 | 179.571±5.9 | 180.100±2.3 | 179.100±3.658 |
| Left ovary weight (g) | 0.033±0.003 | 0.032±0.0 | 0.049±0.025 | 0.045±0.001 | 0.039±0.002 |
| Primordial follicle | 0.500±0.028 | 400±0.025 | 1.250±0.025 | 1.500±0.028 | 1.000±0.040 |
| Primary follicle | 5.750±0.478 | 750±0.440 | 6.250±0.853 | 5.000±0.707 | 5.550±0.886 |
| Secondary follicle | 6.250±0.103 | 220±0.102 | 3.750±0.478 | 6.250±0.478 | 3.750±0.250 |
| Graafian follicle | 9.500±0.645 | 200±0.610 | 8.000±0.135 | 8.000±0.629 | 8.250±0.912 |
| Corpus luteum | 9000±0.147 | 980±0.145 | 7.750±0.085 | 6.000±0.108 | 7.250±0.118 |
| Atretic follicle | 0.000±0.000 | 100±0.010 | 1.500±0.028 | 0.750±0.025 | 1.000±0.040 |

The results are shown as mean ± SD.
The results of the measurement of follicle-stimulating hormone (FSH) levels among different groups showed that the experimental group 1 and experimental group 2 are significantly increased compared to the control group ($P \leq 0.05$), but the experimental group 3 did not change significantly compared to the control group (Figure 1).

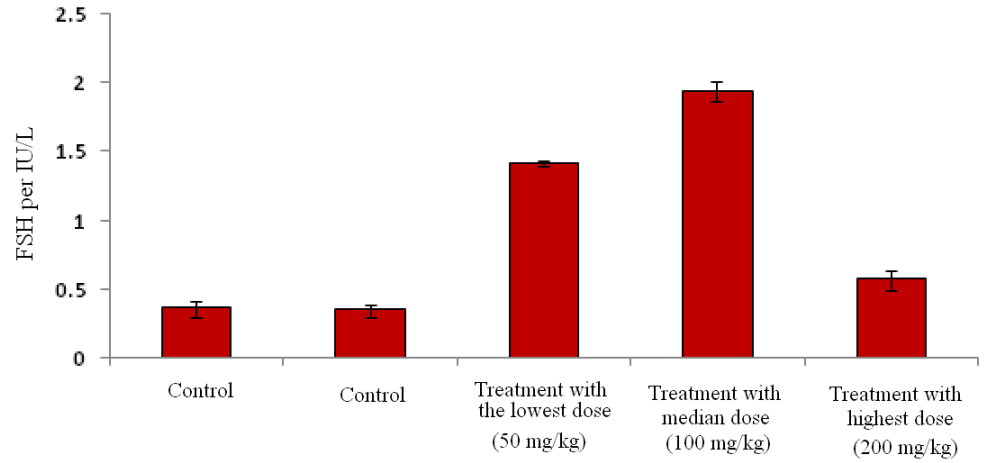


Figure 1: Comparison of follicle-stimulating hormone (FSH) levels in all the groups studied following the effect of pumpkin seed extract

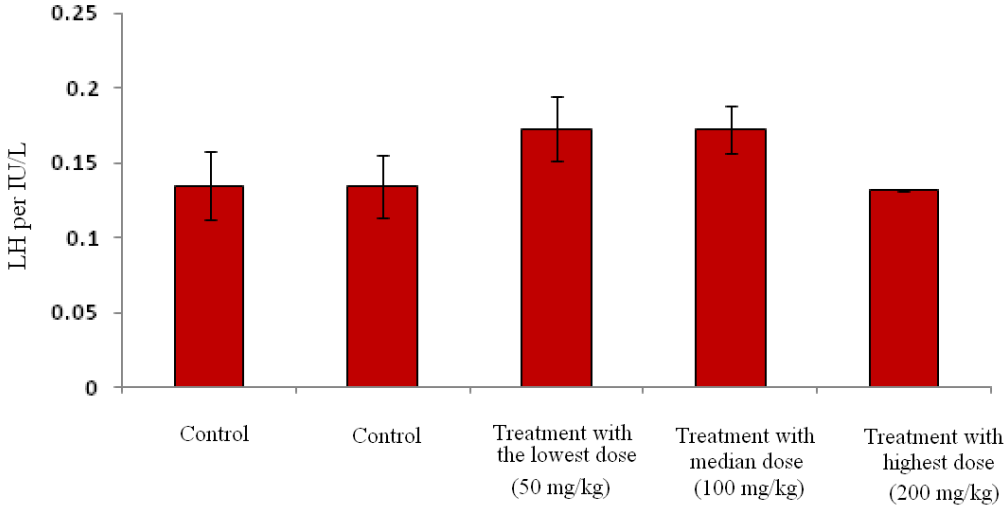


Figure 2 : Comparison of luteinizing hormone levels in all the groups studied following the effect of pumpkin seed extract

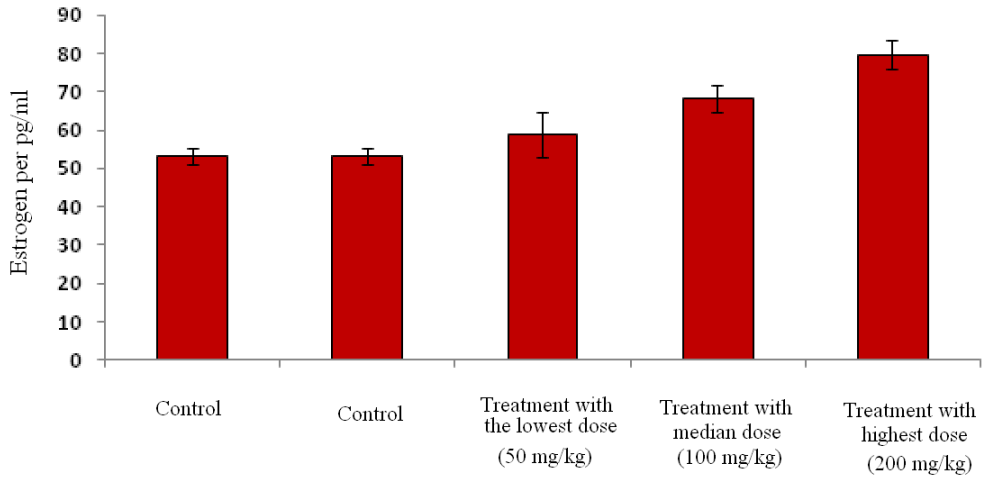


Figure 3: Comparison of estrogen levels in all the groups studied following the effect of pumpkin seed extract

The results of measuring luteinizing hormone levels in different groups showed that the experimental groups 1, 2 and 3 did not change significantly in this regard compared to the control group (Figure 2).

The results of measuring the estrogen levels in different groups showed that in the experimental group 1, this level did not significantly change compared to the control group, but that in the experimental groups 2 and 3, this level significantly increased compared to the control group (P≤0.05) (Figure 3).

The results of measuring the progesterone levels in different groups showed that in the experimental groups 1 and 2, this level did not significantly change compared to the control group, but the experimental group 3 showed significant increase in this regard compared to the control group (P≤0.05) (Figure 4).

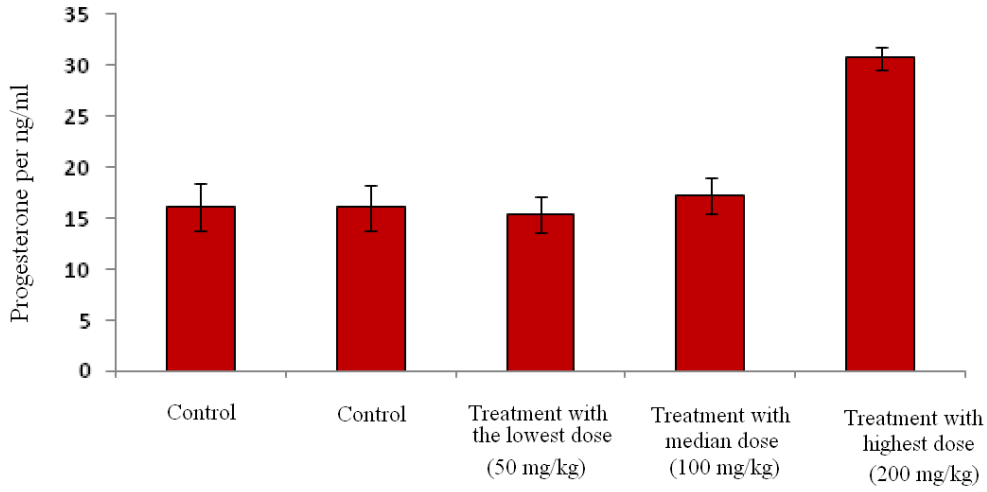


Figure 4: Comparison of progesterone levels in different groups studied following the effect of pumpkin seed extract

## Discussion

The results of this study showed that the injection of hydroalcoholic extract of pumpkin seeds increases estrogen, progesterone and follicle-stimulating hormone (FSH) levels and the number of

secondary follicles and thus results in maturity. This is the first experimental study conducted on immature rats showing the effect of pumpkin seeds on oogenesis and pituitary-ovarian axis.

The consumption of food enriched with pumpkin seeds resulted in a significant weight gain among consumers (21). In the present study, a significant weight gain was observed in the experimental groups receiving 50 and 100 mg/kg of body weight doses.

The study conducted in 2006 by Manal et al. on testicular tissue showed that pumpkin seeds lead to their increase. Reviewing the effect of pumpkin seeds on prostate weight, the binding proteins in prostate cancer and the testicular tissue of rats revealed that in 10% of the cases, pumpkin seeds could inhibit hyperplasia of the ventral lobe of the prostate and also significantly contributed to the reduction of binding proteins in prostate cancer and the improvement of testicular tissue (22). The present study revealed a significant increase in the weight of the left ovaries of the rats.

Successful follicular differentiation depends on the presence of steroid and growth factors stimulating follicular differentiation and protecting the cells against cell death. Ovarian follicles growth and differentiation is achieved through the proliferation and differentiation of granulosa cells. Estrogen plays the main role in the regulation of growth, development, homeostasis, and programmed cell death (apoptosis) in the ovary (23). A 2011 study by Gossell-Williams et al. on postmenopausal women showed that pumpkin seeds are full of phytoestrogen and thus reduce the major symptoms of menopause such as hot flushes, arthraligia and headache (18). Studies conducted by Raicht et al. (1980) and Awad et al. (2000) also showed that pumpkin seeds contain beta-estradiol with its variety of biological effects including reducing cholesterol level, estrogen activity and anticancer activity (24-25). Flavonoids in plants are called phytoestrogen and have estrogen-like qualities (26). The results of the present study showed a significant increase in estrogen in the experimental groups receiving doses 100 and 200 mg/kg of body weight and an increase in progesterone in the experimental group receiving 200 mg/kg of body weight compared to the control group ($P<0.05$), which is consistent with results of the previous studies cited.

For meiosis division to occur and for it to reach the capacity and power of growth and development, the ovule requires appropriate values of the follicle-stimulating hormone and the luteinizing hormone (27). The study conducted by Bataineh et al. showed that pumpkin seed consumption significantly increased serum follicle stimulating hormone and luteinizing hormone in the experimental groups taking pumpkin seed enriched food (21). In the present study, a significant increase was observed in serum follicle-stimulating hormone levels of the experimental group that received 50 mg/kg and 100 mg/kg of body weight doses compared to the control group ($P<0.05$).

In the post maturity phase, high levels of LH and FSH hormones secreted from the anterior pituitary result in the growth and development of the ovary and the ovarian follicles (28), which will be followed by the cascade of events driving ovulation (29). Fatty acids stimulate follicle-making process in terms of the number, size and the growth increase of Graafian follicles (30) and estrogen is a strong follicular growth stimulator in rodents (31). Pumpkin seed is a good source of unsaturated fatty acids and phytosterols (32-34). A study conducted in 1994 by Takada et al. showed that unsaturated fatty acids in the pumpkin seeds that include oleic acid and linoleic acid reduce cholesterol levels in rats (35).

No significant change was observed in the number of primordial and primary follicles in different groups of immature rats

examined in the present study; however, a significant reduction was observed in the number of secondary follicles in the experimental groups 1 and 3 compared to the control group. The contradicting results of the present study compared to those of previous studies could be due to changes in certain important factors such as the animal's breed, hormone levels and duration of treatment. Pumpkin may contain significant amounts of antioxidants, tocopherol, and carotenoids and thus has potential antioxidant activity (36). In their study of 2008, Kim et al. showed the unexpected side effects of antioxidants and stated that the active oxidants present in ovarian follicles are essential for ovulation responses pre-ovulation and inhibit the analysis of ovarian free oxygen species, ovulation and a complete set of essential responses pre-ovulation (37).

The results of the present study are inconsistent with results of the study conducted in 2005 by Forouzanfar et al. as no significant change was observed in the number of Graafian follicles in the ovaries in different groups compared to the control group (30).

Several studies have been conducted on male rats in the past. For example, a study conducted in 2006 by Gossell-Williams et al. showed that pumpkin seeds can inhibit testosterone-induced prostatic hyperplasia and treat this disease (18). A study conducted in 2002 by Dreikorn also showed that pumpkin seeds are useful in the treatment of lower urinary tract symptoms (LUTS) and benign prostatic hyperplasia (BPH) (38). Evidence found in a study conducted in 2007 by Cannon et al. suggests that apoptosis in granulosa cells is the main mechanism involved in ovarian follicle atresia (39). According to a study conducted in 2008 by Kim et al., the side effects of antioxidants include inhibiting ovulation and a complete set of essential responses pre-ovulation (22). The results of the present study indicate a significant increase in the number of atretic ovarian follicles in the two groups of immature rats receiving the maximum and the minimum doses (groups 1 and 3), which is consistent with results of the study by Kim et al. A study conducted in 2000 by Awad et al. showed that pumpkin seeds contain phytoestrogen (40). According to a study conducted in 2003 by Torabizade and Fallahi, a health effect of phytoestrogen is its antioxidant activity (41). The significant increase in progesterone levels in the experimental group 3 might have increased the number of atretic follicles in this group. A study conducted in 1996 by Chun et al. also showed that steroid hormones play an important role in the regulation of cell death in the ovary; estrogens prevent the apoptosis of follicular granulosa cells while androgens contribute to DNA fragmentation in the ovarian cells. These hormones create the mentioned effects by inhibiting and increasing endonuclease activity in the ovarian granulosa cells, in respective order (42).

The results obtained in the present study by counting the corpus luteum of the ovaries in the experimental groups showed no significant change compared to the control group.

Similar studies conducted in the past on other organs, including those from 2005 to 2007, show that snacking on pumpkin seeds provides a good source of zinc and unsaturated fatty acids and phytosterols that are effective on in preventing chronic diseases (10-16,43-44). A study conducted in 2004 by Huang et al. showed that diets containing pumpkin seed significantly decrease the risk of gastric cancer, breast cancer, lung cancer and colorectal cancer (45).

A study conducted in 2011 by Gossell-Williams et al. showed that pumpkin seed consumption causes a significant increase in high-density lipoprotein concentration (46). A study conducted by Suphakarn et al. also showed that the presence of potassium in pumpkin seeds reduces the

risk of calcium oxalate bladder stone formation (47).

## Conclusion

Based on the present study, it can be argued that the hydroalcoholic extract of pumpkin seeds has a positive effect on the ovarian tissue as well as on the pituitary-gonadal axis and leads to significant changes in secondary ovarian follicle and the female sex hormones. Conducting further studies is therefore recommended to find the mechanism of the effect of this substance and its use as a drug effective in starting and regulating oogenesis and successful reproduction.

## Conflict of interests

The authors declare to have no conflicts of interest in this study.

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