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The Effect of Hydro-Alcoholic Extract of Pumpkin Seeds on Estrogen Levels and Kidney Markers in Adult Female Rats

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Abstract

Background: Pumpkin seeds are rich in phytoestrogens (estrogen precursors) and can be good alternatives to synthetic estrogen therapy, without exerting destructive effects on renal and ovarian tissues.
Objectives: In the present study, the effect of hydro-alcoholic extract of pumpkin seeds on estrogen levels was assessed in adult female rats and its effects on serum and tissue markers of kidney and ovaries.
Methods: In this experimental study, 32 adult Wistar female rats (at 60 days of age and weighing10 $\pm$ 180 g) were randomly divided into four groups of 8: three experimental groups and one control group. The three experimental groups received a hydro-alcoholic extract of pumpkin seed via intraperitoneal injection for 21 consecutive days (doses of 20, 50, and 100 mg/kg body weight, respectively). One day after the last injection, blood samples were taken from the rats to test serum levels of blood urea nitrogen (BUN), Creatinine, uric acid (UA), estrogen, progesterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Moreover, their kidneys and ovaries were removed for histological studies.
Results: The results showed significant increases in serum levels of FSH and LH and in the number of graph follicles in the experimental group 3, in addition to significant reduction in the number of primitive and primary follicles in the three experimental groups as compared with those in the control group, in the number of secondary follicles in the experimental group 1 compared with that in the experimental groups 2 and 3, and also in renal cortical thickness, Bowman's capsule thickness and glomerulus diameter in the experimental groups 1 and 2 compared with those in the control group ($P < 0.05$).
Conclousions: It can be concluded that the estrogen found in the hydro-alcoholic extract of pumpkin seeds can play a prominent role in ovulation with the least destructive effect on renal tissue. Hence, it may be a suitable alternative to synthetic estrogen and menstrual cycle regulators.

Keywords: Hormone, Estrogen, Kidney, Rat, Female, Pumpkin Seeds

1. Background

Pumpkins are cultivated worldwide for cooking and medicinal use (1). The cultivation of pumpkins, belonging to the genus of *Cucurbita maxima* and the family of "Cucurbitaceae", began more than 4,000 years ago in South America. The *C. maxima* (Danhobak) species were used in this study. They are annual plants, with meaty fruits and oval-shaped seeds (2-5). Pumpkin seeds are good sources of protein, fat, carbohydrates and minerals (6). The researchers analyzed chemical composition of pumpkin seeds and found linoleic acid, oleic acid and D5-sterol content to be high in the extract (7). A study conducted in 2013 on pumpkin seeds showed crude fiber, crude fat, crude protein and carbohydrate content (to be 31.88%, 31.37%, 33.29%, and 36.35%, respectively), protein, fatty acids, including high contents of saturated fatty acids and the predominant fatty acids including palmitic acid, stearic acid, and linoleic acid. Potassium and sodium are the most prevalent minerals in pumpkin seeds. The α-tocopherol content ranges from 33.33 to 122.65 μg/g (8). Another study in 2018 showed that the main fatty acid found in these seeds is oleic acid (9).

Pumpkin seeds have broad biological effects, including estrogenic activities, anti-cancer activities, and cholesterol-lowering effects. The phytoestrogen (estradiol precursors) extracts isolated from the seeds have estrogenic effects such as prevention of postmenopausal osteoporosis and hyperlipidemia, treatment of hormone-dependent tumors, and reducing main menopausal symptoms. Pumpkin seeds have vitamin E, free radicals (as anti-aging medicine) and antioxidants such as a-tocopherol, g-tocopherol and carotenoids (10-18).

Estrogen has the major role in programmed cell death as well as in regulating ovarian growth, development, and homeostasis. Decreasing estrogen levels are responsible for skin wrinkling in women over the age of 40. Estrogen, when taken early at the beginning of menopause, can maintain the skin structural integrity as well as the vitality of the skin and vessels by increasing collagen. Hormone replacement therapy is also used to treat infertility and low-fertility (19, 20). It should be noted that the use of herbal medicine in different fields has once again attracted the attention of scientists and researchers as a result of drug side-effects, drug intolerance or drug sensitivity, and drug-resistant diseases. Synthetic estrogen is one of the chemical medicines, which affects human renal function (in particular amounts and conditions) (21-23). Hence, the use of pumpkin seeds for hormone replacement therapy can be useful. For this purpose, we should be assured that these seeds have no adverse effects on the human kidney.

Our kidney eliminates metabolic waste materials such as urea, uric acid, creatinine, and ions to maintain the desired chemical composition of body fluid. Blood metabolite concentrations are influenced by increased xanthine oxidase enzyme activity and lipid peroxidation as well as elevated triacylglycerol and cholesterol levels during kidney diseases or kidney damage (24). Pumpkin seeds are excellent sources of protein and pharmaceutical activities such as anti-diabetic, antifungal, antibacterial and anti-inflammatory activities, and anti-oxidant effects (25). The presence of unsaturated fatty acids in pumpkin seeds reduces cholesterol levels in the human body (26) and stimulates kidney function (27, 28). The β-sitosterol, one of the active constituents of pumpkin seeds, improves troublesome of urinary symptoms associated with prostatic enlargement (29). Pumpkin seed oil, alone or combined with phytosterol-F, can increase prostatic weight-to-body weight ratio and protein synthesis and significantly improve BPH/LUTS (30-32).

2. Objectives

Therefore, the present study aimed to investigate the effect of hydro-alcoholic extract of pumpkin seeds on estrogen levels in female adult Wistar rats and its effects on markers of kidney in order to replace synthetic hormone replacement therapy with pumpkin seeds in modern and traditional medicine.

3. Methods

3.1. Animals and Grouping

This basic experimental research was conducted on adult female Wistar rats. These rats are used in the studies investigating ovarian hormones because of the similarity between human menstrual cycle and that of these types of rats (33-37). The rats were taken from the Animal Care Center of Jahrom Islamic Azad University and the practical work was carried out at the same center. The ambient temperature was around 2 ± 23°C (12 hours of light and 12 hours of darkness) with a relative indoor humidity of 40 - 60%. Laboratory animal care principles were correctly implemented (38). All of the experiments were reviewed and approved by the Ethics Committee of Jahrom University of Medical Sciences (code of ethics: jums.REC.1393.071). The sample was selected via simple random sampling and the rats were assigned into groups using the table of random numbers. Thirty-two adult female Wistar rats, at 60 days of age and weighing 10 ± 180 g, were randomly divided into 4 groups of 8, including the experimental groups 1, 2, 3, and the control group. Every day, at 10 AM, the experimental groups and the control group received the right doses of solutions via intraperitoneal injection using insulin syringe for 21 consecutive days (39) as follows:

Group 1: adult rats (weighing 200 grams) received a daily dose of 0.2 mL of pumpkin seed extract (20 mg/kg) (39).

Group 2: adult rats (weighing 200 g) received a daily dose of 0.2 mL of pumpkin seed extract (50 mg/kg) (39).

Group 3: adult rats (weighing 200 g) received a daily dose of 0.2 mL of pumpkin seed extract (100 mg/kg) (39).

Group 4 (Control): received no medicine.

At the end of the study (on day 22), after weighing the animals, blood samples were taken directly from the heart of the animals (anesthetized with hydralazine and lysine) using a 5 cc syringe. A serum centrifuge machine (at 3000 RPM for 15 min (was used for serum preparation and the prepared serum was stored at -20°C.

3.2. Plant Materials, Preparation and Extraction

The Soxhlet method was used for extraction (40). Dry pumpkin seeds (100 g), belonging to the genus of *C. maxima* and "Cucurbitaceae" family and *C. maxima* (danhobak) species (2, 3), were powdered and mixed with 500 mL of 80% ethanol and then stored in a percolator in a lab area for three days. After three days, the extract drops were collected through the percolator valve. The 80% ethanol was added to the machine until the extract was colorless, indicating that there was no extract remained. Then, the obtained clear mixture passed through a filter and the obtained extract was evaporated using a rotary evaporator at a temperature of 40°C until a concentrated extract was obtained. The extract was under vacuum for 24 hours in a desiccator until it was completely dried. Then, the dried extract was weighed and its efficiency was obtained, indicating that 16 g dry extract was extracted from 100 grams of pumpkin seeds powder and the rest was the remaining scum, so the extract was 16%. Finally, the lethal dose and then the maximum, average and minimum doses of the medicine were determined (39).

3.3. Dose Determination

Several concentrations of pumpkin seed extract were randomly selected and injected into 4 groups of 8 rats. The lethal dose (LD50) for the groups in which half of the rats died was 400 mg/kg. The maximum, average, and minimum concentrations were determined. It should be noted that pumpkin seed extract was prepared from the Extraction Center, Shiraz University of Medical Sciences. According to the instructions, there was 400 mg of pure extract in each liter of the prepared extract of which one ml was injected into 5 rats based on their weight (200 g) for LD50 determination. To obtain a dose of 100 mg/kg body weight, the initial extract was diluted four times using physiological serum (experimental group 1). To obtain a dose of 50 mg/kg body weight, the extract was diluted twice using physiological serum (experimental group 2). To obtain a dose of 20 mg/kg body weight, the extract was diluted five times using physiological serum (experimental group 3) (39).

3.4. Measurement of Plasma Biochemical Parameters

Blood samples were used to determine the serum levels of BUN, creatinine, UA, estrogen and progesterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Blood biochemical factors were measured based on the colorimetric method using biochemical test kits, made in Iran, as well as the Selectra XL fully-automated analyzer, made in the Netherlands.

3.5. Microscopic Examination of Renal and Ovarian Tissue

After taking the blood samples, an abdominal incision was made and the kidneys and ovaries were separated from the adipose tissue surrounding them using a scalpel and a forceps. The renal and ovaries of all rats were removed and washed with physiological serum, after being weighed, and were stored in a 10% formalin solution for 14 days. The renal and ovaries were then sent to the histology laboratory of Shahid Motahari Hospital in Jahrom for preparation of the slides.

The slides prepared separately from different parts of renal and ovarian tissues were used for tissue studies. In each slide of the kidney, the diameters of the cortex, medulla, glomerular, Bowman capsules, urinary space, proximal tubule, distal tubule and Henle loop were measured in millimeters. In each slide of the ovaries, hyperemia, vacuolization of the cells of ovarian tissue and follicular atresia, as well as the mean number of primitive, primary, secondary, graph, atretic and yellow follicles, were measured by optical microscope with a magnification of 400× in 10 fields (a total of 50 fields for each animal). Then, the means were determined in each group and compared with those in other groups (39).

3.6. Statistical Analysis

The one-way analysis of variance (ANOVA) was used for data analysis. The Kolmogorov-Smirnov normality test was used to examine if variables were normally distributed and statistical calculations were performed with SPSS software (version 18) at a significance level of 5%. The inclusion criteria were healthy adult female Wistar rats and the exclusion criterion was the death of the animal.

4. Results

4.1. The Effect of Hydro-Alcoholic Extract of Pumpkin Seeds on Serum Concentrations of Female Sex Hormones

There was a significant increase in FSH levels in the experimental group 3 compared with the experimental groups 1 and 2 and the control group ($P < 0.05$). A significant increase in LH levels was observed in the experimental group 3 compared with the control group ($P < 0.05$). A significant increase in estrogen levels was observed in the experimental group 3 compared with the control group ($P < 0.05$) (Figure 1). Progesterone levels did not change significantly in different adult groups ($P < 0.05$).

4.2. The Effect of Hydro-Alcoholic Extract of Pumpkin Seeds on Serum Concentrations of BUN, Creatinine, and UA

No significant changes were observed in creatinine, BUN, and UA levels in the groups which received doses of 20, 50, and 100 mg/kg compared with those in the control group ($P < 0.05$).

4.3. The Effect of Hydro-Alcoholic Extract of Pumpkin Seeds on Kidney

Pumpkin seed extract significantly reduced renal cortical thickness, Bowman's capsule thickness and glomerulus diameter in the groups which received doses of 50 and 100 mg/kg ($P < 0.05$); however, none of the doses had significant effects on the diameters of the central area, Bowman capsules, urinary space, proximal tubule, distal tubule, and Henle loop ($P < 0.05$) (Table 1 and Figure 2).

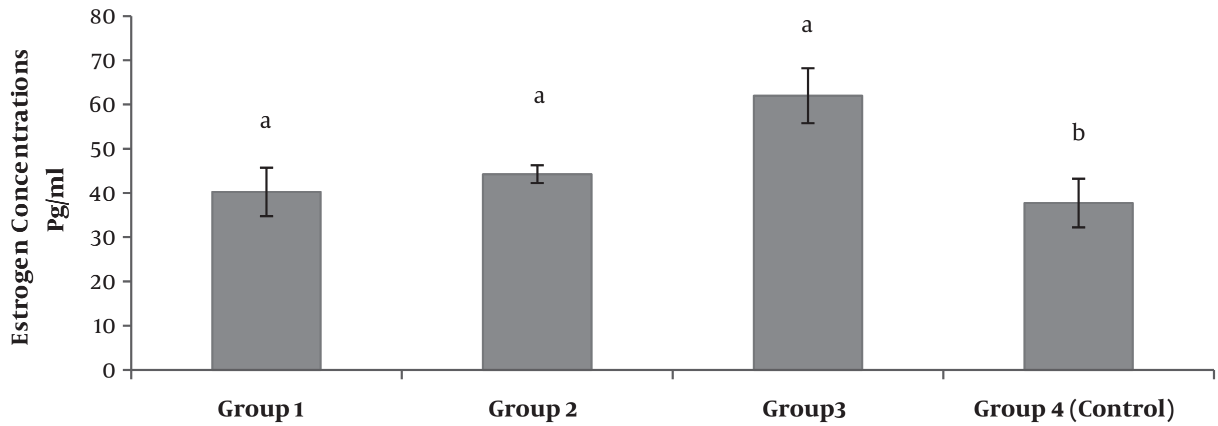

Figure 1. A comparison between different groups for estrogen concentrations (after taking pumpkin seed extract): a significant increase in estrogen levels was observed in the group received a dose of 100 mg/kg compared with other groups. (There was no significant difference between the columns with at least one common letter ($P < 0.05$)). * Significant at $P < 0.05$.

Table 1. Changes in Renal Tissue Variables After Injection of Hydro-Alcoholic Extract of Pumpkin Seeds[a, b, c]

	Group 1	Group 2	Group 3	Group 4 (Control)
Diameters of the cortex, Mm	1007.25 ± 37.83[B]	799.64 ± 12.86[A]	747.73 ± 37.84[A]	1041.46 ± 35.30[B]
Diameter of the Medulla, Mm	2166.129 ± 0.07[A]	2012.188 ± 125.83[A]	1836.135 ± 12.53[A]	2083.39 ± 50.13[A]
Diameter of the glomerular, Mm	367.9 ± 83.62[BC]	343.8 ± 75.81[AB]	339.9 ± 47.15[A]	384.5 ± 41.15[C]
Diameter of Bowman capsules, Mm	438.14 ± 8.50[BC]	406.8 ± 42.58[A]	412.10 ± 77.69[AB]	446.4 ± 74.0[C]
Diameter of urinary space, Mm	71.8 ± 29.65[A]	63.5 ± 58.55[A]	73.5 ± 42.79[A]	62.7 ± 33.75[A]
Diameter of proximal tubule, Mm	178.6 ± 54.59[A]	163.6 ± 47.63[A]	172.5 ± 67.77[A]	183.4 ± 41.56[A]
Diameter of distal tubule, Mm	177.9 ± 41.56[A]	163.5 ± 72.75[A]	160.5 ± 72.52[A]	182.6 ± 20.14[A]
Diameter of Henletubule, Mm	121.5 ± 91.76[A]	110.1 ± 66.45[A]	122.6 ± 50.15[A]	142.5 ± 66.31[A]

[a]Values are expressed as mean ± SD.
[b]Group 1:(received a dose of 20 mg/kg body weight); group 2 (received a dose of 50 mg/kg body weight); group 3: (received a dose of 100 mg/kg body weight); and group 4 (control) ($P < 0.05$).
[c]There was no significant difference between the columns with at least one common letter ($P < 0.05$).

4.4. The Effect of Hydro-Alcoholic Extract of Pumpkin Seeds on Ovarian Tissue

A significant increase was observed in the number of graph follicles in the experimental group which received a dose of 100 mg/kg and a significant decrease was observed in the number of primitive and primary follicles in all three experimental groups compared with the control group. Also, there was a significant decrease in the number of secondary follicles in the experimental group which received a dose of 20 mg/kg compared with the experimental groups which received doses of 50 and 100 mg/kg. No significant changes were observed in the number of secondary, yellow and atretic follicles compared with control group (Table 2).

5. Discussion

Today, synthetic estrogen is used extensively to treat hormonal disorders and infertility among women. In fact, hormone replacement therapy refers to estrogen therapy and sometimes combined estrogen plus progesterone therapy (41). B-estradiol is the major estrogen produced in ovaries. Small amounts of estrone are also secreted; however, the major amounts are produced in peripheral tissues by conversion of the androgens secreted by adrenal cortex and theca cells in the ovary. Estrogen synthesis is directed by FSH and LH hormones, and its synthesis and secretion are increased during the follicular phase of the menstrual period (42-44). Pumpkin seeds contain phytoestrogens with estrogenic activity (13, 14, 45). The phytoestrogens present in human food diet are flavonoids, including Isoflavones. Isoflavones are plant-derived estrogens

Table 2. Changes in Ovarian Tissue Variables After Injection of Hydro-Alcoholic Extract of Pumpkin Seeds[a, b, c]

	Group 1	Group 2	Group 3	Group 4 (Control)
Number of graph follicles, %	10.250 ± 0.850^{AB}	10.500 ± 1.190^{AB}	10.750 ± 1.250^{B}	7.000 ± 1.000^{A}
Number of primitive follicles, %	0.250 ± 0.025^{A}	0.000 ± 0.000^{A}	0.250 ± 0.025^{A}	1.250 ± 0.025^{B}
Number of primary follicles, %	3.000 ± 0.070^{A}	3.250 ± 0.062^{A}	3.075 ± 0.085^{A}	6.000 ± 0.070^{B}
Number of secondary follicles, %	3.000 ± 0.408^{A}	5.280 ± 0.478^{B}	5.000 ± 0.912^{B}	3.750 ± 0.478^{AB}

[a] Values are expressed as mean ± SD.
[b] Group 1 (received a dose of 20 mg/kg body weight); group 2 (received a dose of 50 mg/kg body weight); group 3 (received a dose of 100 mg/kg body weight); and group 4 (control) ($P < 0.05$).
[c] There was no significant difference between the columns with at least one common letter ($P < 0.05$).

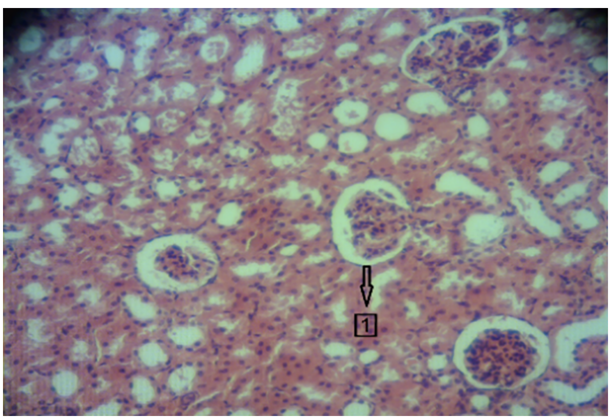

Figure 2. Reduction of Bowman's capsule thickness and glomerulus diameter in group 2 (a dose of 50 mg/kg) compared with those in the control group (hematoxylin and eosin staining at 400× magnification).

binding to estrogen receptors in various organs of the human body, similar to the estrogen in women (46). Steroid hormones have significant effects on gonadal weight and activity. Successful follicular differentiation depends on the presence of steroids and growth factors, which stimulates follicular differentiation and protects cells against cell death. Ovarian follicular growth and differentiation are accomplished by proliferation and differentiation of granulosa cells (19). The oocyte requires adequate FSH and LH for meiotic division and maturation fulfillment (47). In the present study, a significant increase in progesterone and FSH levels was observed in the group 3 (received the maximum dose), which is consistent with the studies conducted by Chaula in 2008. The results of this study showed a significant increase in estrogen levels in the adult rats which received the maximum dose (group 3) compared with the control group. It should also be noted that an increase in LH levels resulted in increased endogen production that is consistent with the studies conducted by Schoultz et al. 1989. In the present study, a significant increase in LH levels was observed in the group 3 (received the maximum dose) compared with the control group.

The cascade of events leading to ovulation is initiated by Follicles. According to previous studies, fatty acids increase the number and size of follicles or promote the growth of graph follicles. As already mentioned, in addition to being good sources of unsaturated fatty acids,9,26 pumpkin seeds have flavonoids with estrogenic potential (phytoestrogens). It should also be noted that estrogen stimulates follicle growth in rodents (33, 46). In the present study, there was a significant decrease in the number of primitive and primary ovarian follicles in different experimental groups compared with the control group and a significant decrease was observed in the number of secondary follicles in the group 1 (received the minimum dose) compared with the groups 2 (received the moderate dose) and 3 (received the maximum dose). However, these results are different from the results of previous studies, probably due to changes in some important factors such as the animal's racial descent, hormone levels, and treatment duration. Maybe the results can be explained by the fact that pumpkins have significant amounts of antioxidants, tocopherols, and carotenoids. Therefore, pumpkins have potential antioxidant activities. The active oxidants present in ovarian follicles are essential for the response to ovulation before ovulation, and the analysis of free ovarian oxygen species prevents ovulation and a complete set of essential responses before ovulation (14-17). The results of the present study showed a significant increase in the number of ovarian graph follicles in group 3 (receiving the maximum dose) compared with the control group which is consistent with previous studies (48).

The kidney is a complex organ with many functions, including removing metabolic waste products and chemicals, regulating water balance and electrolyte homeostasis, arterial blood pressure, acid-base balance, erythropoietin production (stimulating red blood cell production) as well as hormone production, calcitriol production, and generally, blood filtration (49, 50).

The results of previous studies have shown that certain amounts of synthetic estrogen in certain conditions may affect renal function (51). Traditionally, pumpkin seeds have been used as medicines in China, India, Korea, Yugoslavia, Argentina, Brazil, Mexico and the United States to treat intestinal parasites, urinary tract infections, bladder and kidney stones, bile duct and prostate problems. D7-Phytosterolspresent in pumpkin seed oil is useful for the treatment and prevention of prostate, bladder and urinary tract problems (32). Pumpkin seeds also can increase the level of inhibitors precluding crystal formation or accumulation and therefore prevent kidney stone and bladder stone formation. They can also reduce the pressure within the bladder and urinary tract and increase the bladder response rate (52, 53). In fact, pumpkin seeds are diuretic and effective for treating kidney diseases, bronchitis, fever and excessive thirst (28). Studies have shown that one gram of pumpkin seeds contains 28 mg of calcium and 30 mg of phosphorus (54, 55). However, it should be noted that 80% of the phosphorus present in the seeds is stored as phytic acid or phytate which humans cannot digest (56). Phosphorus has a crucial role in bone growth, kidney function, cellular growth and maintaining blood acid-alkaline balance (57). Pumpkin seeds provide high phosphorus levels and can be used as potential agents for lowering the risk of kidney and bladder stones (urinary tract stones) formation (58). In the present study, no significant changes were observed in serum BUN, UA, and creatinine levels in any of the studied groups compared with those in the control group. This indicates that pumpkin seed extract had no adverse effects on renal function.

It should be noted that oxidative stress and lipid peroxidation, resulted from high levels of free radicals, cause damage to the kidney tissue (59). Moreover, decreased activity of endogenous antioxidant enzymes can cause inflammation and destruction of epithelial cells and dilation of renal tubules (60). Pumpkin seeds contain antioxidant molecules, including vitamin A, vitamin E, carotenes, xanthophilic and phenolic compounds which provide protection against oxidative damage (61, 62). Nevertheless, anti-inflammatory and anti-oxidative effects of pumpkin seeds have been broadly established (14, 63-65). Pumpkin seeds also reduce diastolic blood pressure and cause a significant increase in high-density lipoprotein concentrations (18). In this study, the effect of pumpkin seed extract on the kidney tissue was investigated and no change was observed in the central region of the kidney, in proximal tubule diameter, in distal tubule diameter, in Henle loop and urinary space; however, a significant decrease was observed in the diameters of Bowman's and glomerular capsules and the renal cortex in the experimental groups 2 and 3 compared with the control group. The decrease in the diameter of renal cortex was expected due to the decrease in diameters of Bowman's and glomerular capsules, and more damage and pathological changes are justified due to the role of Bowman's and glomerular capsules in the exchange of ions and molecules (66). The factors affecting toxicity include shape and activity of chemicals, effective dose, dose-time relationship, the way of coming into contact with chemicals, chemical species, age, gender, the extent of the chemical's system absorption, distribution and excretion of toxicants in the body and the presence of other chemicals (67). These findings suggest that the hydro-alcoholic extract of pumpkin seeds had the least destructive effect on kidney tissue, mainly affecting the structures of Bowman's and glomerular capsules.

5.1. Conclusions

General conclusion of the present study is that the injection of hydro-alcoholic extract of pumpkin seeds into rats, in addition to increasing estrogen, had positive effects on hormones of the hypothalamic-pituitary-gonadal (HPG) axis and ovarian tissue in estrogen production and surface ovarian follicle production, with the least destructive effect on kidney tissue (just decrease in the diameters of Bowman's and glomerular capsules) and without undesirable effects on serum markers of kidney. These results can be useful for using the estrogen present in pumpkin seeds. It is likely that in the future, conducting molecular experiments and research on pumpkin seeds may allow for alternative solutions instead of synthetic estrogen replacement therapy, oral contraceptives, and menstrual cycle regulators.

Footnotes

Authors' Contribution: Study concept and design: Samaneh Motamed Jahromi and Sadegh Niami. Analysis and interpretation of data: Samaneh Motamed Jahromi. Drafting of the manuscript: Samaneh Motamed Jahromi. Critical revision of the manuscript for important intellectual content: Samaneh Motamed Jahromi. Statistical analysis: Sadegh Niami.

Conflict of Interests: The authors declare that they have no conflict of interest.

Ethical Approval: All of the experiments were reviewed and approved by the Ethics Committee of Jahrom University of Medical Sciences (code of ethics: jums.REC.1393.071).

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