The Effect of Phytosterols and Fatty Acids of Pistachio (Pistacia vera) Oil on Spermatogenesis and Histological Testis Changes in Wistar Adult Male Rats

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Purpose: Oilseeds and their related products are known to have various bioactive and health-promoting ingredients. In this research, we investigated the effects of phytosterols and fatty acids of Pistacia vera on spermatogenesis process and testis histological changes in Wistar male rats for the first time.

Materials and Methods: A total number of 64 adult male Wistar rats were divided randomly into eight groups including one control group, and seven test groups. Test groups received phytosterols, fatty acids, and pistachio oil orally for 30 days. Then, LH, FSH, and serum testosterone levels were determined. Also, the spermatogenesis process and changes in testicular tissue in rats were investigated.

Results: The results of this research suggest that phytosterols in doses of 10 and 50 mg/kg reduce the spermatogenesis process. Fatty acid in a low dose of 10 mg/kg increases spermatogenesis, but when a high dose of 50 mg/kg was used, it harmed the spermatogenesis process. When low levels of phytosterols and fatty acids are used simultaneously in dose 5 mg/kg, improvement in spermatogenesis process is observed but when these were used together in the dose of 25 mg/kg, the spermatogenesis process was disrupted. Using pistachio oil alone also improved spermatogenesis process.

Conclusion: It seems that phytosterols reduce spermatogenesis at high and low doses, while fatty acids increase spermatogenesis when used in low doses and reduce this process when used in high doses. The use of fatty acids extracted from pistachios to treat infertility in men seems hopeful.

Keywords: CMA3; male infertility; short abstinence; sperm DNA integrity; TUNEL

INTRODUCTION

In recent years, one of the problems that human societies have faced is infertility in men[(1)]. Factors involved in male infertility include occupational, environmental, and nutritional factors. Among these factors, diet plays an important role in reproductive health in men[(2)]. Oilseeds are widely used in the human diet; these seeds have many bioactive substances with medicinal and biological properties that have been used in the treatment of several diseases. The evidence suggests that ingestion of Oilseeds may impose different cardiovascular effects thought to be due to their lipid components, which include unsaturated fatty acids, phytosterols and tocopherols[(3)]. Recent research has also shown that dietary intake of edible oil may even have more beneficial effects on total ingested seeds, possibly due to the replacement of carbohydrate diets with unsaturated fats or other oil components[(4)]. Pistachios are one of the most important Oilseeds due to their high-fat content. The most important portion of pistachio fat is unsaturated fatty acids, 80% of which are oleic acid and linoleic acid[(5)].

Pistachio oil has chemical compounds that contain saturated fatty acids such as myristic acid, palmitic acid, stearic acid and unsaturated oils such as linoleic acid, oleic acid, plant sterols and elements such as selenium, zinc, calcium, potassium, iron, and magnesium[(6)]. Previous studies have shown that compounds in pistachio oil inhibit nitric oxide production. Since this compound can control steroidogenesis, therefore, pistachio oil has been used as a drug for the treatment of related diseases, including increased sexual activity[(7)]. On the other hand, it has been reported that plants containing linoleic acid have been used to treat sexual weakness. Saturated and unsaturated fatty acids, for example palmitic acid, oleic acid, linoleic acid, miristic acid and stearic acid inhibit 5-alpha-reductase enzyme activity, which causes the conversion of testosterone to di-hydroxy-testosterone

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Table 1. Body weight of mice in different treated group and control before and after treatment

Body weight (g)a	Before treatment	After treatment
Control (A Group)	203.71 ± 23.26[a]	234.33 ± 15.00[a]
(B Group)	204.14 ± 30.17[a]	217.28 ± 33.50[a]
(C Group)	207.57 ± 41.92[a]	230 ± 38.03[a]
(D Group)	200.57 ± 34.16[a]	237.67 ± 11.64[a]
(E Group)	202.14 ± 40.58[a]	221.63 ± 22.83[a]
(F Group)	205.71 ± 44.12[a]	255.4 ± 17.40[a]
(G Group)	201.86 ± 40.42[a]	224.33 ± 29.45[a]
(H Group)	203 ± 33.98[a]	217.5 ± 44.65[a]
P-Value (Kruskal-Wallis)	.999	.221
P-Value (post hoc)	1	.276

Control group (A), and seven test groups (B to H) were treated orally by gavage for a period of 30 days. Test groups received 10 mg/Kg of phytosterols (B group), 50 mg/Kg of phytosterols (C group), 10 mg/Kg of fatty acids (D group), 50 mg/Kg of fatty acids (E group), 5 mg/Kg of fatty acids plus 5 mg/Kg of phytosterols (F group), 25 mg/Kg of fatty acids plus 25 mg/Kg of phytosterols (G group) and 50 mg/Kg of pistachio oil (H group). Mean values in a column followed by different letters are significantly different ($P < 0.05$)

(DHT), so inhibiting its activity increases the amount of testosterone in the blood[8]. Non-saturated fatty acids can inhibit 5-alpha-reductase enzyme activity, and saturated fatty acids increase cholesterol[9].

Sterols are a group of natural compounds that are derived from hydroxylation of polycyclic isopentanoids and have a structure of 1, 2-cyclopentanophenanthrene. Most plant sterols contain 28 or 29 carbon and have one or two carbon-carbon double bonds in their molecular structure, one of which is dual bonds inside the rings, and the other is on the side chain of the sterol structure. More than 200 different types of plant sterols have been reported in plant species. Five sterols including β-sitosterol, Δ5-avenasterol, campesterol, stigmasterol and cholesterol have been identified in pistachio oil[10].

In recent years, considerable attention has been paid to the study of the effect of different plants on fertility of laboratory mammals. Therefore, this study aimed to investigate the effect of the extracted phytosterols and fatty acids from pistachio (Pistacia vera var. Akbari) oil on spermatogenesis and changes in testicular tissue in adult male Wistar rats and also possibly to predict any benefits or harms of these compounds on fertility.

MATERIALS AND METHODS

Plant preparation

The fruits of Pistacia vera var. Akbari were collected

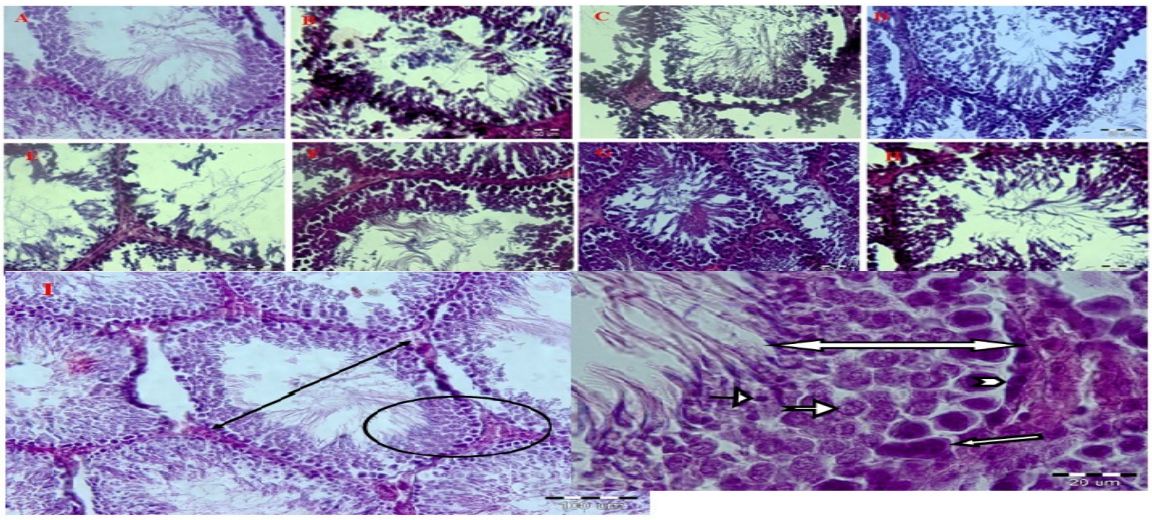

Figure 1. Histological sections of the testes of mice in different treated groups and control.
Control group (A), and seven test groups (B to H) were treated orally by gavage for a period of 30 days. Test groups received 10 mg/Kg of phytosterols (B), 50 mg/Kg of phytosterols (C), 10 mg/Kg of fatty acids (D), 50 mg/Kg of fatty acids (E), 5 mg/Kg of fatty acids plus 5 mg/Kg of phytosterols (F), 25 mg/Kg of fatty acids plus 25 mg/Kg of phytosterols (G) and 50 mg/Kg of pistachio oil (H), respectively and (I) demonstrates high magnification photomicrograph obtained from testis of rat showing:
Sertoli cells
Spermatogonia cells
Spermatocyte cells
spermatide cells
Epithelial thickness
Seminiferous diameter

Table 2. The testis weight of mice in different treated groups and control after treatment

Testis weight (g)[a]	After treatment	Testis weight/BW
Control (A Group)	1.35 ± 0.1[b]	0.005761 ± 0.00052[a]
(B Group)	1.31 ± 0.07[b]	0.006029 ± 0.0013[a]
(C Group)	1.34 ± 0.15[b]	0.005826 ± 0.00088[a]
(D Group)	1.31 ± 0.15[b]	0.005512 ± 0.0007[a]
(E Group)	1.33 ± 0.16[b]	0.006255 ± 0.0013[a]
(F Group)	1.44 ± 0.17[a]	0.005638 ± 0.00061[a]
(G Group)	1.16 ± 0.18[b]	0.005171 ± 0.0015[a]
(H Group)	1.25 ± 0.13[b]	0.005747 ± 0.0018[a]
P-Value(Kruskal-Wallis)	.212	.843
P-Value(post hoc)	.119	.815

Control group (A), and seven test groups (B to H) were treated orally by gavage for a period of 30 days. Test groups received 10 mg/Kg of phytosterols (B group), 50 mg/Kg of phytosterols (C group), 10 mg/Kg of fatty acids (D group), 50 mg/Kg of fatty acids (E group), 5 mg/Kg of fatty acids plus 5 mg/Kg of phytosterols (F group), 25 mg/Kg of fatty acids plus 25 mg/Kg of phytosterols (G group) and 50 mg/Kg of pistachio oil (H group). Mean values in a column followed by different letters are significantly different ($P < 0.05$)
[a]Data are presented as mean ± SD

from a garden in Rafsanjan, Iran and approved by a botanist at the Vali-e-Asr University of Rafsanjan.

Reagents

All chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification except when mentioned specifically.

Pistachio oil preparation

The oil of the pistachio kernels was obtained by cold-pressing the dried kernels. The oil was protected from direct sunlight and stored at 4-6 °C.

Preparation of phytosterol fraction

Unlike fatty acids, phytosterols cannot saponify. Therefore, phytosterols were separated from fatty acids using their reactions with NaOH. To extract the sterols, 0.1 g of the pistachio oil was mixed with 20 mL of 1 M ethanolic NaOH solution and stirred for 12 h at room temperature. Then, 20 mL distilled water and 40 mL diethyl ether were added to the mixture. In this step, the obtained mixture was transferred to a decanter where the sterols were separated from the saponified fatty acids and transferred into the ether phase. After, separating the sterol-rich ether phase, the extraction of the remained sterols from the aqueous phase was using 40mL of excess diethyl ether. The two ether phases were mixed and transferred to a decanter where possible saponifiable components were removed by extracting with 0.5 M ethanolic NaOH solution. The sterol-rich ether phase was finally freeze-dried and the solvent-free sterols were stored at -20 °C until use[11].

Preparation of fatty acid fraction

To extract the fatty acid fraction, 0.1 g of the pistachio oil was mixed with 20 mL ethanolic NaOH solution (1 M) and stirred for 12 h at room temperature. Then, the mixture along with 20 mL distilled water and 40 mL diethyl ether was transferred to a decanter. The saponified fatty acids were dissolved in water activating the aqueous phase. After separating the aqueous phase, 40 mL of NaOH solution (0.5 M) was added and the extraction procedure was carried out again to extract the remaining saponified fatty acids from the ether phase. The separated aqueous phases were mixed and reacted with 20 mL HCl solution (0.5 M) to convert the saponified fatty acids to free fatty acids. After adding

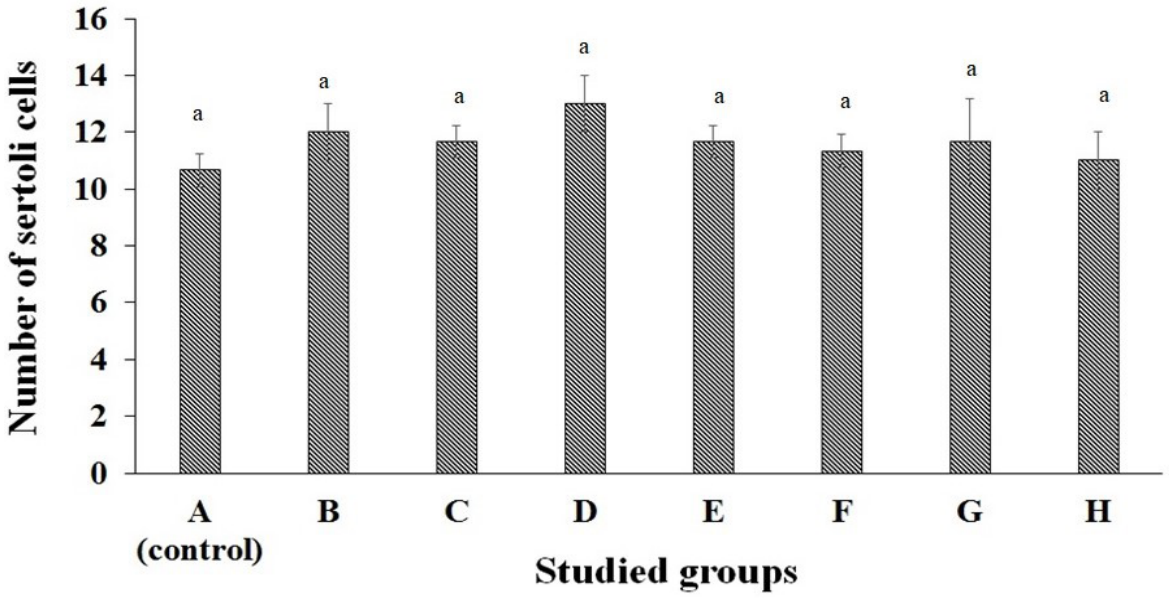

Figure 2. The number of sertoli cells of mice fed on different concentrations of phytosterols and fatty acids of pistachio oil

Table 3. The mean serum testosterone, FSH and LH levels in different treated groups and control after treatment.

Parametersa Groups	Testosterone (ng/mL)	FSH (mlu/dL)	LH (mlu/dL)
Control (A group)	1.53 ± 0.08[c]	0.05 ± 0.003[a]	0.42 ± 0.04[a]
(B group)	0.73 ± 0.05[e]	0.06 ± 0.008[a]	0.39 ± 0.02[a]
(C group)	0.68 ± 0.06[e]	0.07 ± 0.005[a]	0.44 ± 0.05[a]
(D group)	1.93 ± 0.04[b]	0.05 ± 0.006[a]	0.36 ± 0.02[a]
(E group)	1.41 ± 0.7[d]	0.08 ± 0.009[a]	0.34 ± 0.01[a]
(F group)	1.45 ± 0.09[d]	0.06 ± 0.004[a]	0.45 ± 0.07[a]
(G group)	1.51 ± 0.08[c]	0.05 ± 0.001[a]	0.40 ± 0.08[a]
(H group)	2.12 ± 0.12[a]	0.07 ± 0.002[a]	0.97 ± 0.02[b]
P-Value(Kruskal-Wallis)	.002		
P-Value(post hoc)	<.001		

Control group (A), and seven test groups (B to H) were treated orally by gavage for a period of 30 days. Test groups received 10 mg/Kg of phytosterols (B group), 50 mg/Kg of phytosterols (C group), 10 mg/Kg of fatty acids (D group), 50 mg/Kg of fatty acids (E group), 5 mg/Kg of fatty acids plus 5 mg/Kg of phytosterols (F group), 25 mg/Kg of fatty acids plus 25 mg/Kg of phytosterols (G group) and 50 mg/Kg of pistachio oil (H group). Mean values in a column followed by different letters are significantly different ($P < 0.05$)
[a]Data are presented as mean ± SD

40 mL diethyl ether, the free fatty acids were extracted to the ether phase from the aqueous phase. This procedure was repeated again and finally, the separated ether phases were mixed, freeze-dried and stored at -20 °C until use[12].

Experimental assays

The present study was performed on 64 adult male Wistar rats, weighing 200±45 g that were kept in the animal laboratory of Rafsanjan University of Medical Sciences. In this experimental study, the animals were housed at room temperature (25 °C), and light was set at 12 h light–dark cycle. They were maintained in plastic cages separately and had free access to food and water. The study protocol was approved by the Ethical Committee of Rafsanjan University of Medical Sciences under the ethical code IR.RUMS.REC.1396.98.

They were randomly divided into eight groups (n = 8) including one control group (A), and seven test groups (B to H). Test groups received 10 mg/Kg of phytosterols (B group), 50 mg/Kg of phytosterols (C group), 10 mg/Kg of fatty acids (D group), 50 mg/Kg of fatty acids (E group), 5 mg/Kg of fatty acids plus 5 mg/Kg of phytosterols (F group), 25 mg/Kg of fatty acids plus 25 mg/Kg of phytosterols (G group) and 50 mg/Kg of pistachio oil (H group) orally by gavage for 30 days. At the end of the treatment period, the animals were anesthetized with ketamine (70 mg/kg) and xylazine (10 mg/kg) mixture. Then, rats were killed by cervical dislocation and testicular tissues were used for the histological analysis.

Hormones and biochemical assays

Enzyme immunoassay test for serum testosterone, FSH and LH were performed according to manufacturer instructions. Testosterone, FSH and LH were determined by using kits purchased from AccuBind ELISA Kit, Monobind, USA.

Histological analysis

For histological investigations, testes were quickly dissected and weighed immediately after killing the rats, then divided into small pieces and placed in 10% paraformaldehyde (PH = (7.2) for 72 h for fixation. For each group, testis tissue samples of four rats were selected

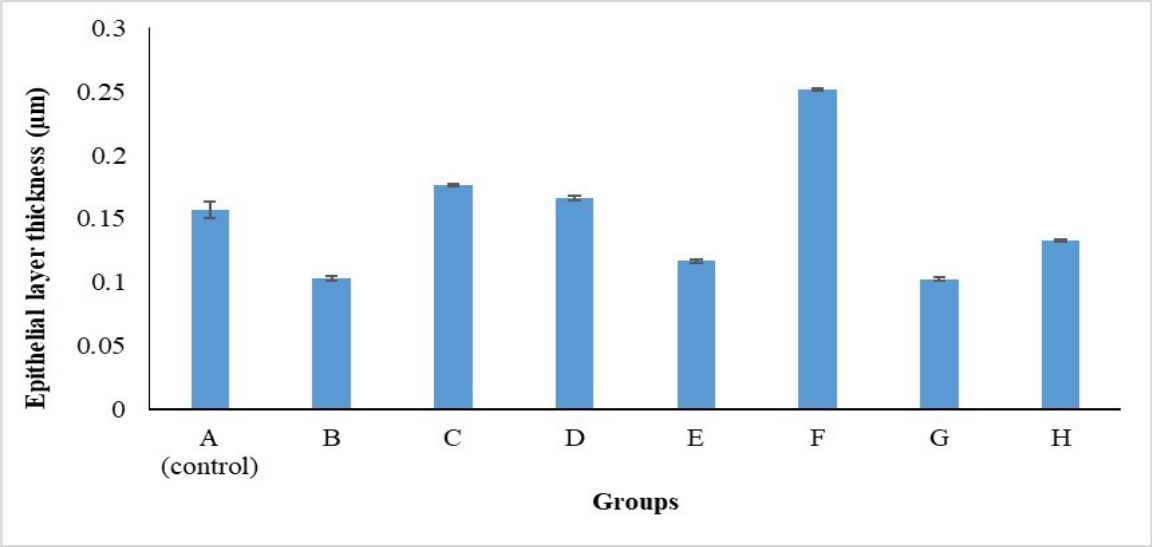

Figure 3. The result of epithelial layer thickness of mice fed on different concentrations of phytosterols and fatty acids of pistachio oil
Data are presented as mean ± SD. Bars with same superscript letters are not significantly different whereas those with different superscript letters are significantly different ($p < 0.05$).

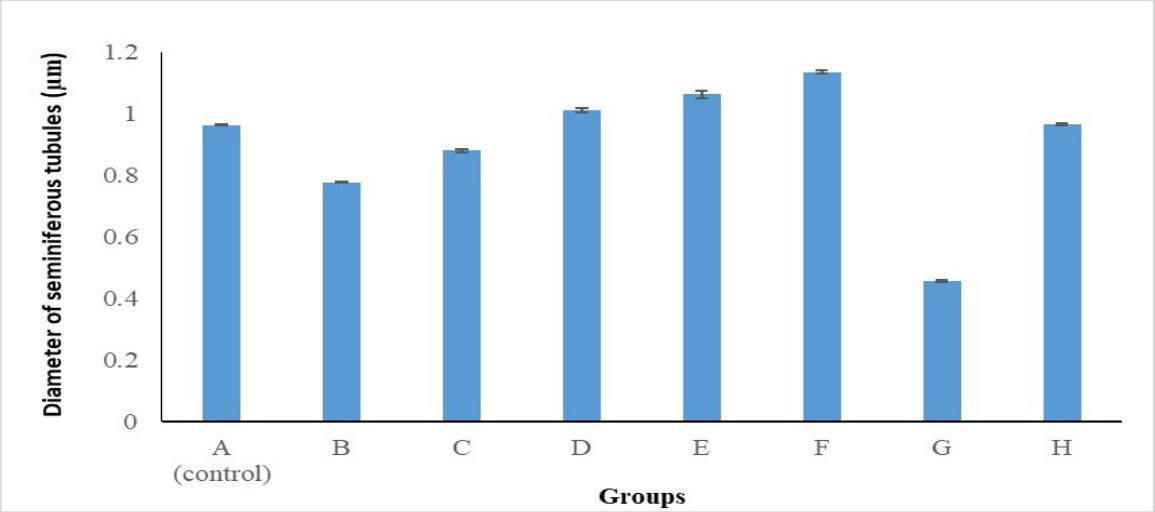

Figure 4. The diameter of seminiferous tubules of mice fed on different concentrations of phytosterols and fatty acids of pistachio oil Data are presented as mean ± SD. Bars with same superscript letters are not significantly different whereas those with different superscript letters are significantly different ($p < 0.05$).

randomly, then ten tissue samples were selected from each testis by a systematic random sampling protocol. Tissue samples were directly dehydrated in a graded series of ethanol, cleared in xylen, impregnated in paraffin wax and embedded in paraffin block. From each sample, thin sections with a thickness of 5 µm were prepared by a rotary microtome machine, and finally 5 sections were randomly selected from each sample using the systematic uniform random sampling. All collected sections on slides were stained with hematoxylin-eosin (H & E) and were observed under an optical microscope (E-200, Nikon, Japan). Then the microscopic fields of the slides were photographed randomly with a Nikon camera and the images were transferred to a computer and analyzed with Image tool software. The number of sertoli, spermatogonia, spermatocyte, spermatide cells as well as epithelial thickness and seminiferous tube diameter were determined with image tool software and statistically compared in all studied groups.

Statistical analysis

Normality of the data was tested by Kolmogorov-Smirnov method. Results are expressed as Mean ± SD. The difference between groups was compared using one-way ANOVA. Also, post hoc approach was used to compare groups with control. If significant difference was detected, multiple comparisons were made using the Tukey-HSD ($\alpha = 0.05$). All the statistical analyses were carried out using the SPSS.26 software. A *p*-value below 0.05 was considered statistically significant.

RESULTS

This study has attempted to investigate the effect of pistachio (Pistacia vera) oil with phytosterols and fatty acids extracted from it on spermatogenesis and testis histological changes in Wistar male rats and possibly to predict any benefits or harms of these compounds on fertility.

Body and testis weight

The effects of pistachio oil with phytosterols and fatty acids extracted from it were investigated on body weight in male Wistar rats. The mean body weights of mice in all test groups increased but no observed significant change before and after 30 days' of treatment ($P = .999$ and $P = .221$, respectively). Also, the results of the present study revealed that the body weight in all test groups no had significant value in comparison to the control group ($P = .276$ and $P = 1$, respectively) (**Table 1**).

The oral administration of phytosterols and fatty acids extracted from pistachio oil resulted in an increase in testis weight in the F group that the rats received 5 mg/kg phytosterol + 5 mg/kg fatty acid, but this increase is not significant compared to the control group ($P = .119$). In addition, the ratio of testis weight to body weight in test groups showed no significant difference in comparison to the control group ($P = .815$) (Table 2). An increase in the testis weight is related to the number of spermatids and sperm present in the tissue.

Serum LH, FSH and testosterone levels

Administration of pistachio oil, phytosterols and fatty acids in different concentrations for 30 days had no significant effect on serum level of FSH hormone in Wistar male rats($P = .06$). But the serum level of testosterone was decreased significantly ($P = .002$) in rats who received phytosterols in 10 mg/Kg and 50 mg/Kg concentrations (B and C groups, respectively) compared to the control group ($P = 0$). Also, the serum level of this hormone was increased in D and H groups including rats receiving 10 mg/Kg of fatty acids and 50 mg/Kg of pistachio oil, in comparison to the control, respectively ($P = 0$). The serum level of LH was unchanged between all test groups and the control group except for the H group that increased after treatment with 50 mg/Kg of pistachio oil ($P = .04$) (**Table 3**).

Histological examinations

Figure 1 shows the tissue sections in all groups under study. According to the results of the histological examination of testes in all groups of study, the mean number of sertoli cells was not significantly changed in all treated groups compared to control group (**Figure 2**).

The epithelial layer thickness in all experimental groups

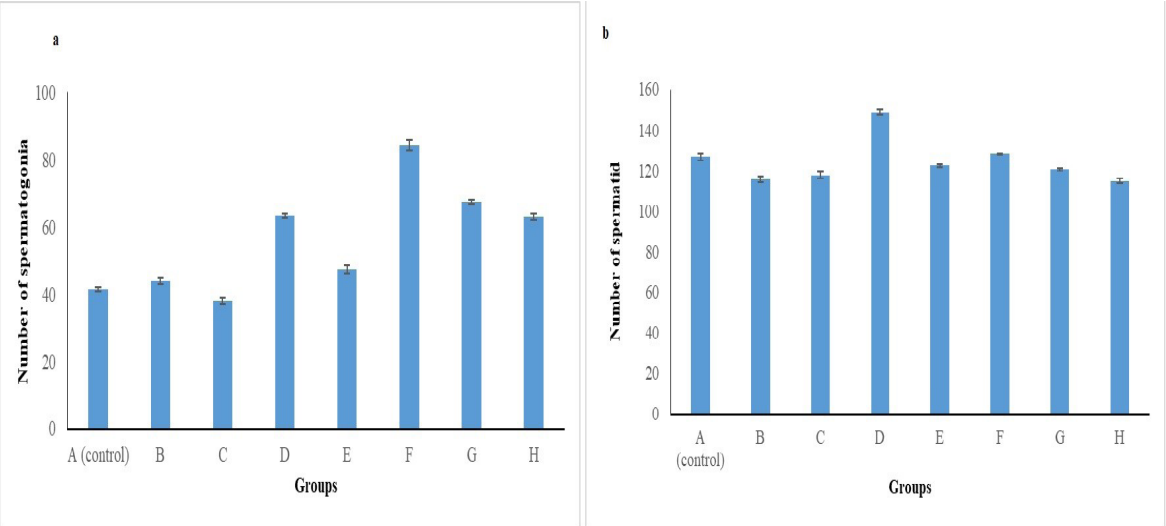

Figure 5. The number of spermatogonia (a) and spermatid (b) of mice fed on different concentrationw of phytosterols and fatty acids of pistachio oil, respectively.
Data are presented as mean ± SD. Bars with same superscript letters are not significantly different whereas those with different superscript letters are significantly different ($p < 0.05$).

was significantly different from the control group ($P = 0$). According to the results of the present study, the epithelial layer thickness was significantly decreased in B, E and G groups that received 10 mg/Kg of phytosterols, 50 mg/Kg of fatty acids and 25 mg/Kg of fatty acids plus 25 mg/Kg of phytosterols, respectively. While the epithelial layer thickness in C, D, F and H groups that received 50 mg/Kg of phytosterols, 10 mg/Kg of fatty acids, 5 mg/Kg of fatty acids plus 5 mg/Kg of phytosterols and 50 mg/Kg of pistachio oil, respectively were significantly increased compared to the control group (**Figure 3**).
The diameter of seminiferous tubules was significantly decreased in the G group while in other groups it did not significantly change (**Figure 4**).
The morphological findings of the study and the spermatogonia count that used tissue sections as well as the comparison between the mean number of spermatogonia showed that there was a significant difference in spermatogonia number, between all test groups and the control group except for the B group that remained unchanged after treatment with 10 mg/Kg of phytosterols. So that the number of spermatogonia was significantly decreased in the C group that received 50 mg/kg of phytosterols, while the number of spermatogonia in D, E, F, G and H groups that received 10 mg/kg of fatty acids, 50 mg.kg fatty acids, 5 mg/Kg of fatty acids plus 5 mg/Kg of phytosterols, 25 mg/Kg of fatty acids plus 25 mg/Kg of phytosterols and 50 mg/Kg of pistachio oil, respectively, were significantly increased compared to control group (**Figure 5a**).
The number of spermatids was significantly decreased in B, C, E and G groups that received 10 mg/Kg of phytosterols, 50 mg/Kg of phytosterols, 50 mg/Kg of fatty acids and 25 mg/Kg of fatty acids plus 25 mg/Kg of phytosterols, respectively. While the number of spermatid in D, F and H groups that received 10 mg/Kg of fatty acids, 5 mg/Kg of fatty acids plus 5 mg/Kg of phytosterols and 50 mg/Kg of pistachio oil, respectively were significantly increased compared to control group (**Figure 5b**).

DISCUSSION

Pistacia, a genus of flowering plants from the family Anacardiaceae, contains about twenty species, among them five are more commonly recognized, including *P. vera*, *P. atlantica*, *P. terebinthus*, *P. khinjuk*, and *P. lentiscus.* Different parts of Pistacia species have been used in traditional medicine for various aims like aphrodisiac[13]. Various types of phytochemical constituents like terpenoids, phenolic compounds, fatty acids, and sterols have also been isolated and identified from different parts of Pistacia species[13]. Pistacia species have oleaginous fruits considered by several researchers. The oil content in P. vera kernel is about 50–60% [14,15]. The main fatty acid in kernel of P. vera is oleic acid[16,17]. Other fatty acids identified in these species are linolenic, palmitic, palmitoleic, stearic, myristic, eicosanoic, behenic, lignoceric, arachidonic, pentadecanoic, hexadecanoic, octadecanoic, and margaric acid[14]. The most abundant sterol reported in fruits of *P. vera* is β-sitosterol followed by Δ5-avenasterol, campesterol and stigmasterol[15].
The present study aimed to investigate the effect of the extracted phytosterols and fatty acids from pistachio (Pistacia vera var. Akbari) oil on spermatogenesis and changes in testicular tissue in adult male Wistar rats and also possibly to predict any benefits or harms of these compounds on fertility.
In the F group that the rats received 5 mg/kg phytosterols + 5 mg/kg fatty acids, the thickness of epithelium layer and also the amount of spermatid and spermatogonia increased. In general, spermatogenesis process improved. In contrast, in the G group that the rats received 25 mg/kg phytosterol+ 25 mg/kg fatty acid, the thickness of the epithelium layer decreased, the diameter of tubules decreased and despite increased spermatogonia cells, the number of spermatids was decreased, resulting in a general decrease in spermatogenesis process. In the H group that received pistachio oil, the thickness of the epithelium layer, the number of spermatogonia and spermatids and the overall spermatogenesis process increased.

Phytosterols extracted from pistachio oil decrease the cholesterol desmolase c activity by reducing the conversion of cholesterol to bergenolone in mitochondria, thereby reducing the synthesis of steroids including testosterone.

All phytosterols have anti-androgenic effects and decrease tissue sensitivity to androgens, besides, androgen activity is decreased by inhibiting 5-alpha reductase Inhibition of this enzyme reduces the conversion of testosterone to dihydrotestosterone, an active form of this hormone in tissues[18]. In addition, phytosterols can treat benign prostatic hyperplasia (BPH) in the prostate by reducing testosterone and dihydrotestosterone levels.

Phytosterols also decrease the level of steroid hormones such as testosterone by lowering cholesterol levels[19].

Compounds in pistachio oils such as zinc and linoleic acid can inhibit nitric oxide production. These compounds can inhibit steroidogenesis, so pistachio oil probably increases steroidogenic function in Leydig cells through inhibition of the synthesis of nitric oxide and consequently increases the concentration of testosterone[7].

In conclusion, the results of this study suggest that phytosterols in doses 10 and 50 mg/kg reduce spermatogenesis process. Fatty acid in a low dose of 10 mg/kg increases spermatogenesis process, but when a high dose of 50 mg/kg was used, it had a negative effect on spermatogenesis process. When low levels of phytosterols and fatty acids are used simultaneously in dose 5 mg/kg, improvement in spermatogenesis process is observed but when these were used together in high dose of 25 mg/kg, the spermatogenesis process was disrupted. Using pistachio oil alone also improved spermatogenesis process. It seems that phytosterols reduce spermatogenesis process and fatty acids increase spermatogenesis when used in low doses and reduce this process when used in high doses.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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