Assessment of eruca sativa leaves extract ZnO NPs effect on the adverse effects of creatine - induced testes injury

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Abstract

This study was conducted to evaluate the protective role of Eruca sativa NPs on the harmful effects of creatine on the testes in male rats. This study used 48 male rats, in two experiments. The first experiment included determining the most effective nano-concentration of watercress extract, while the second experiment included evaluating the role of the nano-extract at the concentration that was chosen based on the results of the first experiment (60 mg/kg) against toxicity resulting from doses of creatine (4 g/kg) for 60 days. This study used 24 adult male rats were randomly assigned to four groups, with six animals per group, and were treated as follows:(G1) 1 ml of 0.09% physiological solution , (G2) dosed (single dose/day) with nano-extract of Eruca sativa at a concentration of (60 mg/kg) , (G3) The animals were dosed (single dose/day) with creatine monohydrate (4 g/kg), (G4 prevention group) dosed with nano-extract of Eruca sativa (60 mg/kg) followed one hour later by a dose of creatine (4 g/kg) . The results of the study indicated a non significant in Concentration of sperm in the epididymis, Percentage of live sperm ,Percentage of motile sperm , Percentage of normal sperm, LH , FSH and Testosterone in G2 and G4 compared to G1. As for the preventive group G4, it recorded a normal results near from control group. it recorded a significant decrease ($P<0.05$) in Concentration of sperm in the epididymis, Percentage of live sperm ,Percentage of motile sperm , Percentage of normal sperm, LH , FSH and Testosterone in G3 compared to G1 and G2 . The results of the histological examination in G3 showed that there are interstitial spaces between the seminiferous tubules and few sperm in the cavities of the tubules with reduction and disintegration of the interstitial tissue and a lack of Leydig cells and a decrease in the size of the germinal epithelial cell layer with cell degeneration necrosis of cells lining the tubules compared to G1,G2 and G4. The results of this article revealed the positive effects of Eruca sativa leaf ZnO NPs extract on the adverse effects of creatine-induced testes injury.

Keywords: *Eruca sativa*, creatine, nano-zinc oxide, testes injur.

Introduction

Creatine monohydrate supplements are among the most popular nutritional supplements in the world [1] . Although they have been extracted since 1832, creatine supplements did not become mainstream or known to people until the 1990s, when two Olympic gold medalists announced that Part of the credit for their athletic success was their intake of creatine supplements [2].

Then it became so popular that surveys indicated that about 80% of the athletes who participated in the 1996 Atlanta Olympic Games used creatine to generate energy [3,4]. Since then, there has been a wide range of research on the effectiveness and safety of implementing a diet containing creatine supplements, especially after the first doubts about the safety of using these supplements appeared in 1998, after three wrestlers died while preparing for competition after consuming this supplement. Centers for Disease Control and Prevention (1997) (In the middle of the year reported a case associated with taking these supplements with symptoms of weight loss, fatigue and shortness of breath in football players [5].

Johnson et al., (2012)[6] presented a study that aimed to investigate the mechanisms behind the effect of creatine monohydrate and its role in causing testicular toxicity in rats. The research included dosing mice with creatine monohydrate and examining changes in testicular oxidative stress through markers and activities of antioxidant enzymes. They found that creatine monohydrate led to increased oxidative stress and decreased antioxidant enzyme activities in the testicles. The study concluded that creatine monohydrate, which causes testicular toxicity in rats, is associated with increased oxidative stress and weak antioxidant defense. A study was also conducted by [7] on Laboratory animals examined the effect of creatine monohydrate on possible testicular oxidative stress, apoptosis, and mitochondrial function. The study revealed that creatine monohydrate stimulates oxidative stress, disrupts mitochondrial function, and triggers apoptosis pathways in testicular cells [8] also confirmed that creatine monohydrate induces testicular toxicity. Through experiments the researchers conducted on male mice and analyzed changes in signs of oxidative stress and antioxidant enzyme activity. The results indicate that dosing with creatine monohydrate leads to an imbalance between oxidative stress and antioxidant defense mechanisms, resulting in testicular damage. Some studies indicate that high doses or prolonged use of creatine may lead to adverse effects on testicular parameters such as sperm count, motility, and shape. However, controversy over creatine toxicity on the testicles continues, with factors such as dose concentration, duration of use, and variation in response being important considerations [9].

Treatment with medicinal plants occupied a large place in medical and pharmaceutical sciences and became a safe source for the pharmaceutical industry at the beginning of the current century, as attention was paid to using them in the programs of the World Health Organization despite the great development in the fields of chemistry, pharmacy

and the chemical drug industry, as it indicated Recent studies have shown the effect of medicinal plants as alternative antioxidants to medicines and chemical treatments. Recent scientific studies and research have proven the medicinal effectiveness of many plant compounds that possess the properties of antioxidants that reduce the severity of some diseases or protect against them, especially cancer in humans, in addition to a lower rate of disease risk [10, 11].

Some medicinal plants contain effective chemicals of high benefit and importance due to their effective role in improving biochemical parameters and their therapeutic activity in the organs of the human and animal body [12], leaves of the watercress plant contain a percentage It is high in active compounds, which are carbohydrates by 28%, proteins by 36%, tannins by 14.3%, glycosides by 6%, alkaloids by 11%, and saponins by 7.4%. The percentage of humidity rises in the leaves to reach about 90.7%. This high percentage of humidity works to reduce the concentration of toxic substances, especially the presence of acid. Erucic acid, and therefore the leaves can be used as a food substance for humans, in addition to containing secondary metabolic compounds with high biological activity, such as flavonoids and phenols[13,14,15].

The Eruca sativa plant also contains several mineral elements such as potassium, calcium, aluminium, manganese, iron, copper, sodium, nickel, cadmium, and zinc, with a high nitrogen content [16], in addition to being rich in vitamins in the plant's seeds and leaves such as Niacin, Biotine, and B12. , K, A, E, and C [17]. The watercress plant is also characterized by containing sulfur glycosides, such as glucosinolate compounds, which contain the sulfur element, which is the reason for giving this plant its distinctive smell [17].

It also contains several flavonoids (Flavonoidesm Kaempferol 3-O-(2 O-malonyl-ß-D-glucopyranoside)-4 O-ß-D glucopyranoside, rhamnocitrin 3-O-(2"-O-methylmalonyl-ß-D). -glycopyranosid)-4'-O-ßD-glucopyra-[noside glucopyranoside, kaempferol 3, 4 di-O-glucopyranoside, 3-O-glucopyranoside, 4'-O-[glucopyranoside, rhamnocitrin 3-O-glucopyranoside, kaempferol and rhamnocitrin Which are powerful antioxidants [18].

In a study presented by Grami etal., [19], male rats were dosed with aqueous extract of watercress leaves and its effect on the male reproductive system and fertility. To study the ameliorative activity of E. sativa aqueous extracts at doses of 50, 100, and 200 (mg\kg .B.W.) on reproductive toxicity associated with oxidative stress caused by Bisphenol A , the simultaneous administration of the two lower doses led to improvement of all histological and biochemical parameters. Their study indicated the ability The extract helps prevent testicular damage in rats, which may be related to functional bioactive substances such as phenolic compounds with high antioxidant capacity.

ZnO-NPs supplementation to rabbits showed a significant increase in ejaculate volume, sperm viability, and sperm motility. No information is available regarding [20]. Effect of nano-zinc oxide supplementation on semen quality and fertility of male rabbits. In a study presented and compiled by [21], the number of sperm in the nano-zinc oxide group increased significantly ($P < 0.001$) compared to those in the group treated

with diorubexin. This is a predictable result given that testosterone levels are so high. Testosterone is an androgen in the testicle that promotes spermatogenesis. As a stimulant of antioxidants. Their results were also supported by improvement of histopathological changes in the testis, testis. Improvement in the structure of the seminiferous tubules, preservation of the shape of sperm and sperm cells, and treatment of atrophy of the seminiferous tubules caused by the ingestion of benzo[α]pyrene by rabbits, which interferes with the process of sperm formation [22].

Materials and Methods

This study was conducted at the animal facility of the College of Education of Pure Sciences, University of Karbala. Ethical permission was obtained from the research and experimental group College of Education Ethics Committee for Pure Sciences - Department of Life Sciences, under Ethical Approval No. 326 in November 2023. Male white laboratory Wistar Albino Rats were used in the study. They numbered (48) rats, their weights ranging from 200-250 grams and their ages (10-12 weeks), which were brought from the animal house of the College of Pharmacy - University of Karbala.

The study included two experiments. The first included determining the concentration of the nano-extract of eruca sativa that was most effective from three ascending concentrations (20, 40, 60 mg/kg) that are safe for use. Its 24 rats for a period of 30 days, while the second experiment included evaluating the role of the nano-extract at the concentration that was chosen according to the results of the first experiment (60 mg/kg) against the toxicity resulting from dosing with creatine at a concentration of 4 g \kg

Experiment 1

The initial study involved a cohort of 24 fully-grown male rats weighing between 200 and 250 grams. These rats were separated into four groups of identical size and administered specific doses for a duration of 30 days, as outlined below:

1- Control group: The saline nutrient solution was dosed during the experiment.

2- The first extract group: dosed orally with nano- *Eruca sativa* extract (20 mg/kg) after dissolving it in 1 ml of distilled water.

3- The second extract group: dosed orally with nano- *Eruca sativa* extract (40 mg/kg) after dissolving it in 1 ml of distilled water.

The third extract group: dosed orally with nano- *Eruca sativa* extract (60 mg/kg) after dissolving it in 1 ml of distilled water.

Experiment 2

Based on the results of the first experiment, a concentration of 60 mg/kg of nano-ZnO extract of watercress leaves was approved to evaluate its effectiveness against hepatic, renal, and reproductive toxicity resulting from exposure to creatine monohydrate (4 g/kg) for a 60 days. The experimental animals were distributed in a number of 24 adult rats were randomly assigned to four groups, with six animals per group, and were treated as follows:

1- Control group (G1): 1 ml of 0.09% physiological solution was dosed throughout the experiment.
2- Group dosed with nano-extract of *Eruca sativa* (G2): The animals were dosed (single dose/day) with nano-extract of *Eruca sativa* at a concentration of (60 mg/kg) after dissolving it in 1 ml of distilled water.
3- Creatine group: (G3) The animals were dosed (single dose/day) with creatine monohydrate (4 g/kg) after dissolving it in 2 ml of distilled water.
4- The preventive group dosed with nano-extract of *Eruca sativa* before creatine (G4): This represents the prevention group, and its animals were dosed (single dose/day) with nano-extract of *Eruca sativa* (60 mg/kg) after dissolving it in 1 ml of distilled water, followed one hour later by a dose of creatine (4 g/kg) after dissolving it in 2 ml of distilled water.

Preparation of plant extract

The plant extract was prepared according to [23] with some modifications. It was prepared from fresh *Eruca sativa* found in the markets by collecting the leaves and washing them with tap water to get rid of the dirt stuck in them. Then, they were washed with distilled water several times, dried in the shade, and ground using an electric grinder to obtain the powder is sifted and stored in a container in the shade. Weigh 5 grams of it and place it in a glass beaker and add 400 milliliters of distilled water to it, Stir constantly and leave for 12 hours. Then, it is filtered using several layers of gauze. The resulting solution is collected and placed in test tubes. For the centrifuge device at speed (1200) rpm. In order to get rid of the remaining biological materials and fibers, the filtrate is dried in the oven at a temperature of 40 degrees Celsius to produce a greenish-brown powder and stored in an opaque glass bottle at a low temperature.

Preparation of nano-zinc oxide extract

Zinc oxide nanoparticles were synthesized using the method described according to [24], with certain alterations. The procedure involved the addition of 6 grams of aqueous extract powder to 100 milliliters of distilled water in a 500 milliliter glass beaker, followed by the addition of 0.1 grams of zinc acetate. To achieve a pH of 7, add diluted ammonia to each 100 ml of solution while continuously stirring the mixture with a magnetic stirrer operating at 200 rpm. Maintain the temperature at 37 degrees Celsius for 24 hours until the color changes from dark green to dark green. The solution was further purified by filtration using No. 1 (Whatman) filter paper. Subsequently, the contaminants were isolated using centrifugation at a speed of 4500 revolutions per minute for a duration of 30 minutes. The resulting sediment was then thoroughly rinsed twice with distilled water and subsequently dried at a temperature of 50 degrees Celsius. The gray powder of zinc oxide nanoparticles is obtained by subjecting it to high temperatures in an oven.

Results and Discussion

Results of diagnosing the nano-extract of watercress by atomic force microscopy

The results of the current study showed the process of revealing the nature of the surface of the generated nanoparticles, which showed the surface roughness of the nanoparticles as well as the shape and size of the generated particles and the extent of their agglomeration, as in Figure (1). The results of the analysis by atomic force microscopy (AFM) showed that the average size of the nanoparticles reached (53.12 nm) and the surface height according to the 3D image was (10.69 µm/div) and the average surface roughness was (10.44 nm).

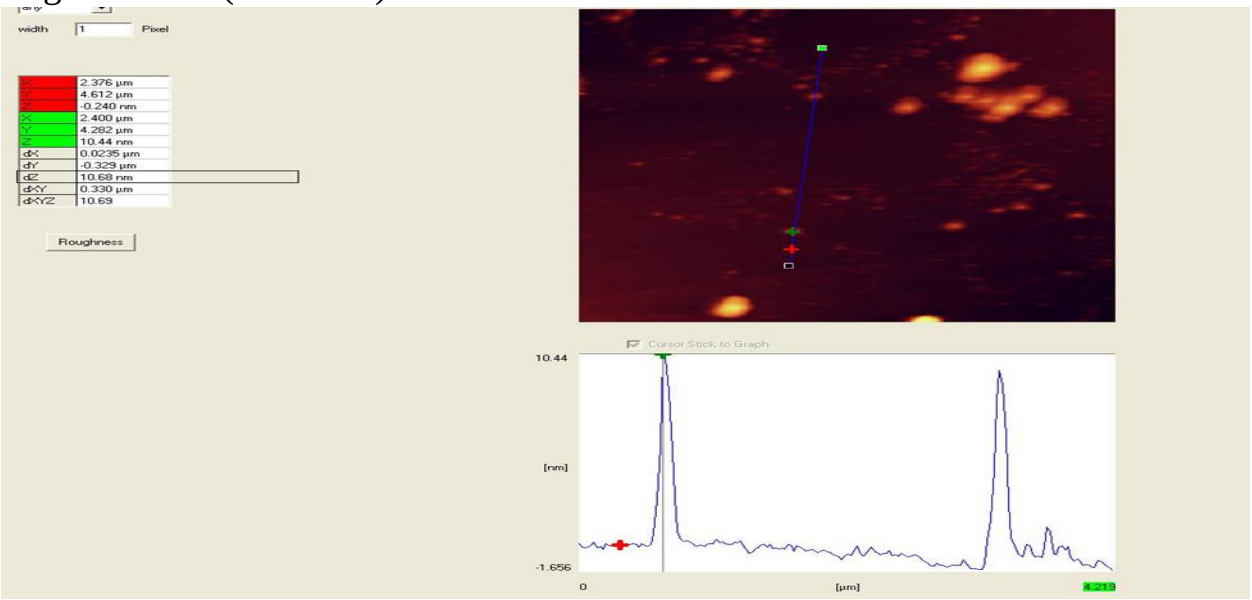

Figure (1): AFM analysis of ZnO nanoparticles manufactured from aqueous extract of watercress.

Sperm parameter results

Sperms count of epididymis

The results of the current study showed that there was no significant difference (P≤0.05) in the sperm concentration (88.16 ± 1.60) compared to the control group (89.16 ± 0.98) and a significant decrease (P≤0.05) in the percentage of sperm concentration in the creatine group (88.16 ± 1.60) compared to the control group had a significant increase (P≤0.05) in the total protective watercress nano extract 72.66 ± 4.03 compared to the G3 and a significant decrease (P≤0.05) compared to the control group.

Percentage of live sperms

The current study also recorded that there were no significant differences (P≤0.05) in the percentage of live sperm in the watercress nano extract group, 86.66 ± 2.06, compared to the control group, 86.16 ± 1.16, and a significant decrease (P≤0.05) in the percentage of live sperm G3, 58.16 ± 3.97.

Compared to the control group G1, it recorded a significant increase (P≤0.05) in its percentage in the preventive group dosed with watercress nano extract with creatine,

72.00 ± 3.57 compared to the creatine group, 58.16 ± 3.97, and a significant decrease (P≤0.05) for the same group compared to the control group.

Percentage of motile sperms

The results of the study also showed that there were no significant differences (P≤0.05) in the percentage of motile sperm in the G2 comparative with G1, but a significant decrease (P≤0.05) was recorded the percentage of motile sperm in the G3 creatine group was compared to G1. The G4 preventive group also recorded a significant increase (P≤0.05) (69.66 ± 5.16) compared to the G3 .

Percentage of normal sperms

The results of the current study showed that there was no significant difference (P≤0.05) in the percentage of vital sperm in the group dosed with watercress nano extract 88.00 ± 1.09 compared to the control group 90.50 ± 1.87.

A significant decrease (P≤0.05) in the group dosed with creatine, 56.66 ± 3.34 compared to the control group, and a significant increase (P≤0.05) in the percentage of normal sperm in the preventive group, 71.16 ± 1.94, compared to the group dosed with creatine, and a significant decrease (P ≤0.05) compared to the control group.

Table (1): The protective role of nano-extract of Eruca sativa leaves (60 mg/kg) against creatine-induced toxicity on some sperm parameters in male rats.

Group	Concentration of sperm in the epididymis (10^6×)	Percentage of live sperm%	Percentage of motile sperm %	Percentage of normal sperm%
Control	89.16 ± 0.98 A	86.16 ± 1.16 A	83.83 ±1.72 A	90.50 ± 1.87 A
Eruca sativa leaves extract ZnO NPs (60mg/kg)	88.16 ± 1.60 A	86.66 ± 2.06 A	83.16 ± 2.63 A	88.00 ± 1.09 A
Creatine (4 gm/kg)	56.33 ± 2.42 C	58.16 ± 3.97 C	44.50 ±4.67 C	56.66 ± 3.34 C
Eruca sativa leaves extract ZnO NPs (60mg/kg)+ Creatine (4 gm/kg)	72.66 ± 4.03 B	72.00 ± 3.57 B	69.66 ± 5.16 B	71.16 ± 1.94 B
LSD	3.0507	3.5215	4.6057	2.6885

The results of our current study in the decrease in sperm concentration and vitality and the rate of motile and normal sperm in the group dosed with creatine agreed with

[25] and agreed with a study presented by [26 ,27] on laboratory animals and examining the effect of creatine. Monohydrate potentially affects testicular oxidative stress, apoptosis, and mitochondrial function. The study revealed that creatine monohydrate stimulates oxidative stress, disrupts mitochondrial function, and triggers apoptotic pathways in testicular cells.

This decrease in sperm concentration may be attributed to the decrease in the secretion of the hormones FSH and LH from the anterior lobe of the pituitary gland, which was also recorded in our study in Table (2), which leads to the inhibition of testosterone production, which affects the process of sperm formation and thus a decrease in the concentration of sperm inside the tubules. Carrier of sperm and fertility of animals. The production of naturally mature sperm is the basis of male fertility [25]. The process of producing sperm and the hormone Testosterone in the testicle is regulated mainly by the hormones FSH and LH, which are released from the pituitary gland and are the main regulators of the process of sperm formation [28] that a decrease in the level of reproductive gonad nutrients may have prevented the initiation of sperm formation in treated animals, because suppressing gonad nutrients prevents the signal responsible for initiating and completing the process of sperm formation during the natural maturation of the developing or developing sperm, as well as initiating and maintaining the process of sperm formation. Quantitatively and qualitatively, naturally, it requires the presence of sufficient levels of the gonadal hormones and Testosterone. Insufficient levels of these hormones are usually associated with severe abnormalities in the sperm and thus may lead to a state of oligospermia or asthenia. In addition to that, the function of the accessory gonads also depends on The presence of sufficient levels of Testosterone in the blood circulation [29].

The reason for the high rate of sperm concentration in the groups dosed with nano-watercress extract before creatine was due to the effect of the active substances collected in the leaves of the watercress plant, such as phenolic substances, since the watercress plant contains glucosinolates in addition to other components of phenolics, such as polyphenols and fatty acids that act as antioxidants and protect the cell from Damage, as these results were in agreement with [14,19] and the reason could be due to the fact that watercress acts as a diuretic, as watercress works to activate the kidneys to increase the excretion of urine after it has been treated, as well as Its preventive role, and then works to reduce the level of urea and creatinine.

The protective role of nano extract Eruca sativa leaves (60 mg/kg) against creatine-induced toxicity on some hormones in male rats.

Our current study recorded no significant differences ($p<0.05$) in the levels of follicle-stimulating hormone (0.270 ± 0.01), luteinizing hormone (0.152 ± 0.004), and testicular adipose hormone (4.90 ± 0.41) in the group dosed with nano-watercress extract, and its decrease in the hormones mentioned in the creatine group above. (0.251±0.007), (0.142±0.004), and (1.73±0.29), respectively, compared with the control group (0.261±0.007), (0.151±0.007) and (4.65±0.14), respectively.

As for the preventive group dosed with nano-arugula extract before creatine, no significant differences were recorded in the rates of FSH levels, 0.251 ± 0.004, and LH, 0.146 ± 0.004.

Compared to the control group and the creatine group, as for the testicular lipid hormone, it recorded a significant increase in the preventive group by 3.19 ± 0.03 compared to the creatine group until it returned to the level recorded in the control group (0.14 ± 4.65).

Table (2): The protective role of nano-extract of Eruca sativa leaves (60 mg/kg) against creatine-induced toxicity on some hormones in male albino rats.

Group	FSH	LH	Testosterone
Control	0.261 ± 0.007 AB	0.151 ± 0.007 AB	4.65 ±0.14 A
Eruca sativa leaves extract ZnO NPs (60mg/kg)	0.270 ± 0.01 A	0.152 ± 0.004 A	4.90 ± 0.41 A
Creatine (4 gm/kg)	0.251 ± 0.007 B	0.142 ± 0.004 C	1.73 ±0.29 C
Eruca sativa leaves extract ZnO NPs (60mg/kg)+ Creatine (4 gm/kg)	0.251 ± 0.004 B	0.146 ± 0.004 BC	3.19 ± 0.03 B
LSD	0.0103	0.0062	0.3209

The reason for the decrease in the level of the FSH and LH in the creatine group, as well as the decrease in the level of testosterone in the blood, which was in agreement with [30] due to the creatine substance, is either a direct effect of the supplement on the Leydig cell or an indirect effect through Disturbances of the hormonal environment in the pituitary-pituitary axis, which causes inhibition of the release of the hormones LH and FSH and reduces their levels, thus reducing testosterone [31].

Testosterone is one of the most important androgens secreted and manufactured by interstitial cells, also known as Leydig's cells. These cells are under the influence and control of LH secreted from the anterior part of the pituitary gland, and because of its effect on Leydig's cells, it is called interstitial stimulating hormone in males. (Interstitial cells stimulating hormone [32]. The hormone testosterone plays the main role in the manifestation of secondary sexual characteristics, as well as the activity and development of the male reproductive system. It also has a decisive role in regulating and stimulating the process of spermatogenesis and spermiogenesis, as a low level of testosterone (T) is one of the causes of weakness. Fertility in men [33].

The results of our current study agreed with [19, 34] who studied the potential protective effects of the ethanolic extract of watercress leaves on the reproductive system of adult male rats exposed to hydrogen peroxide. The researchers used forty adult male

rats and were treated daily for 60 days. An increase was seen in the level of the same hormones under study, in addition to other physiological parameters. The reason for the increase in the hormones FSH, LH, and testosterone in the preventive groups dosed with watercress extract with a creatine supplement is attributed to the role of watercress extract in modifying The decline occurring in the levels of reproductive hormones in the blood of rats treated with creatine to the extent that there was no significant difference recorded from their levels in the control group is attributed to the effectiveness of the extract in improving hormone levels to its role in stimulating Leydig cells to produce the hormone testosterone through its direct action on testicular tissue, in addition to its role in reducing Creatine causes hormonal disruption at the level of the hypothalamic-pituitary-gonadal axis [34].

This is due to the presence of sterols, flavonoids, quercetin, and saponin in watercress extract, which have the ability to scavenge and displace free radicals to improve fertility and testicular functions, and thus, increase sexual desire. A study showed that the presence of glucosinolates (the main glucosinolate is Erucin) and other stimulant substances found in the watercress plant led to many biological activities, including the ability to protect cells from oxidative stress and improve the characteristics of semen [35].

Histological changes in testicular tissue : Microscopic examination of histological sections taken from the testicles of animals of the control group (Figure 2) and the group treated with the nano extract (Figure 4) showed the normal structure of the testicular tissue, which consists of round or oval seminiferous tubules that are well and regularly arranged. Each tubule is surrounded by a basement membrane lined with sperm-generating cells, between which are spread a group of Sertoli cells with triangular nuclei. Spermatogenic cells include spermatids, which appeared as small round cells resting on the basement membrane, spermatocytes, which were larger with dark, round nuclei, and spermatocytes, which appeared round or oblong near the lumen. The cavities of the tubules appear filled with sperm, and the interstitium is observed filling the spaces between the tubules and containing Leydig cells and blood vessels.

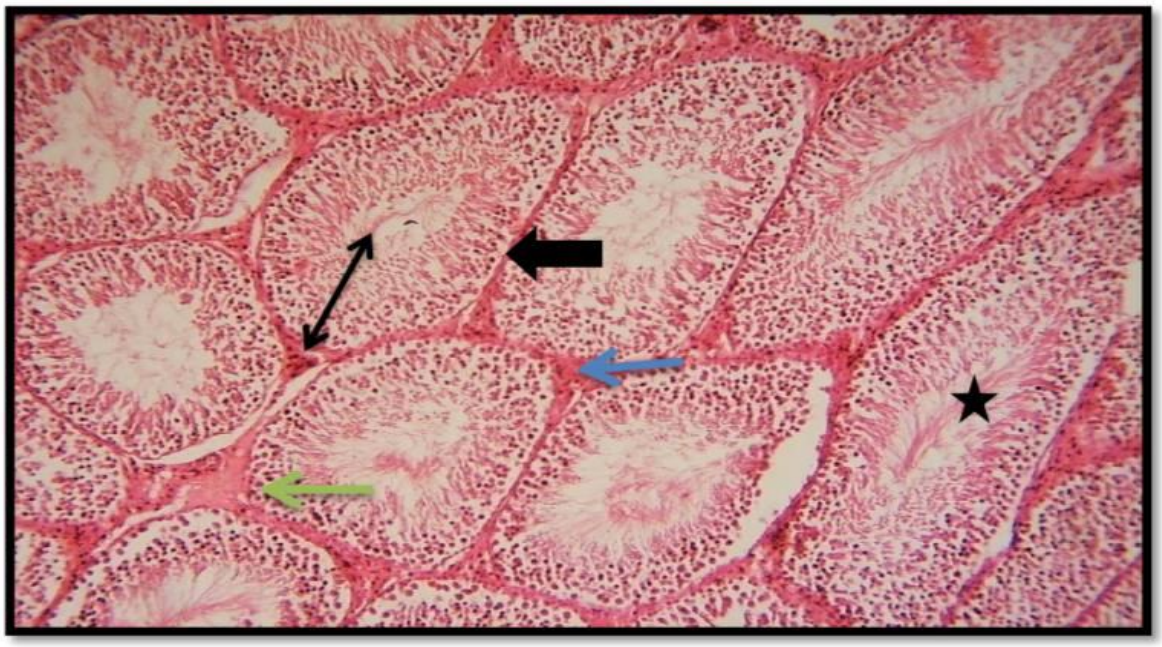

Figure (2): a cross-section of the testicle of a rat from the control group, in which normal tissue of the testicle is observed with the seminiferous tubules ( ) and their cavities filled with sperm ( ) with Leydig cells in the interstitial tissue ( ) and the germinal epithelial cell layer ( ) and sperm progenitors ( ) (H & E 100X).

The group treated with creatine (Figure 3) showed a distorted structure of the testicular tissue, characterized by widening of the spaces between the seminiferous tubules, disintegration of the germinal layer and a lack of connection between spermatogenic cells and Sertoli cells, separation or detachment of the germinal layer from the basement membrane in most of the tubules. The cavities of some tubules are devoid of sperm and their quantity decreases in the cavities of others, the number of sperm cells and Sertoli cells decreases, atrophy of some tubules so that they appear small and separate as a result of the disintegration of the connective tissue, and they also show a widening of the cavity as they are empty or almost devoid of sperm.

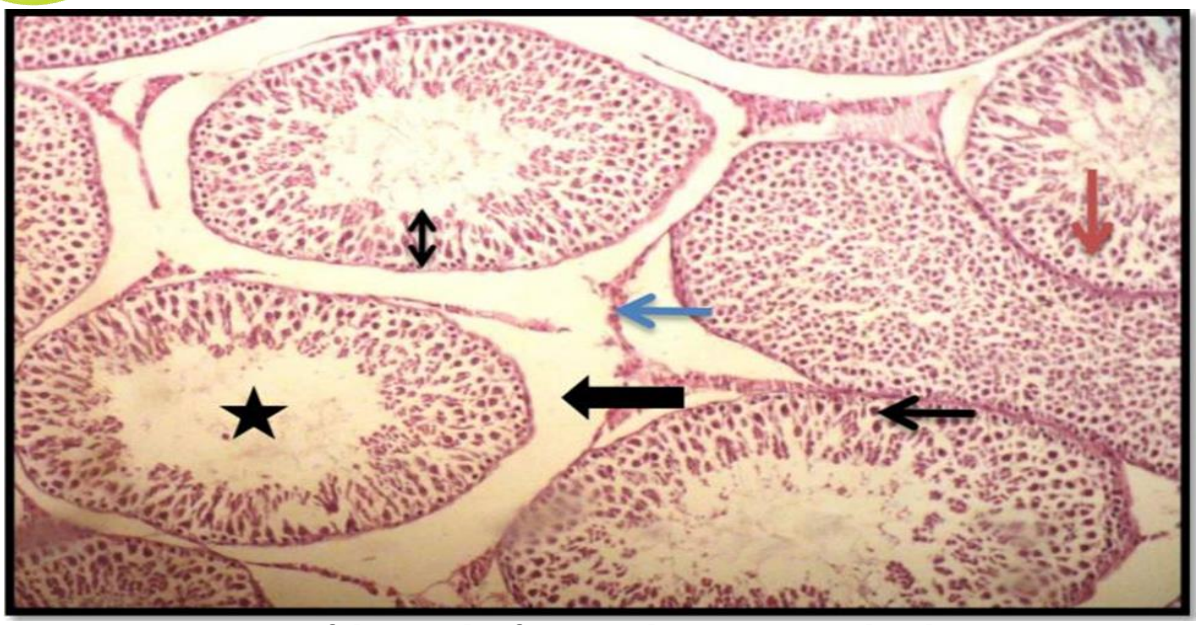

Figure (3): a cross-section of the testicle of a rat in the group treated with creatine at a concentration of 4 g/kg body weight, in which it is noted that there are interstitial spaces between the seminiferous tubules (⬅) and few sperm in the cavities of the tubules (★) with reduction and disintegration of the interstitial tissue and a lack of Leydig cells (←) and a decrease in the size of the germinal epithelial cell layer (↔) with cell degeneration (←) necrosis of cells lining the tubules (←) (H & E 100X).

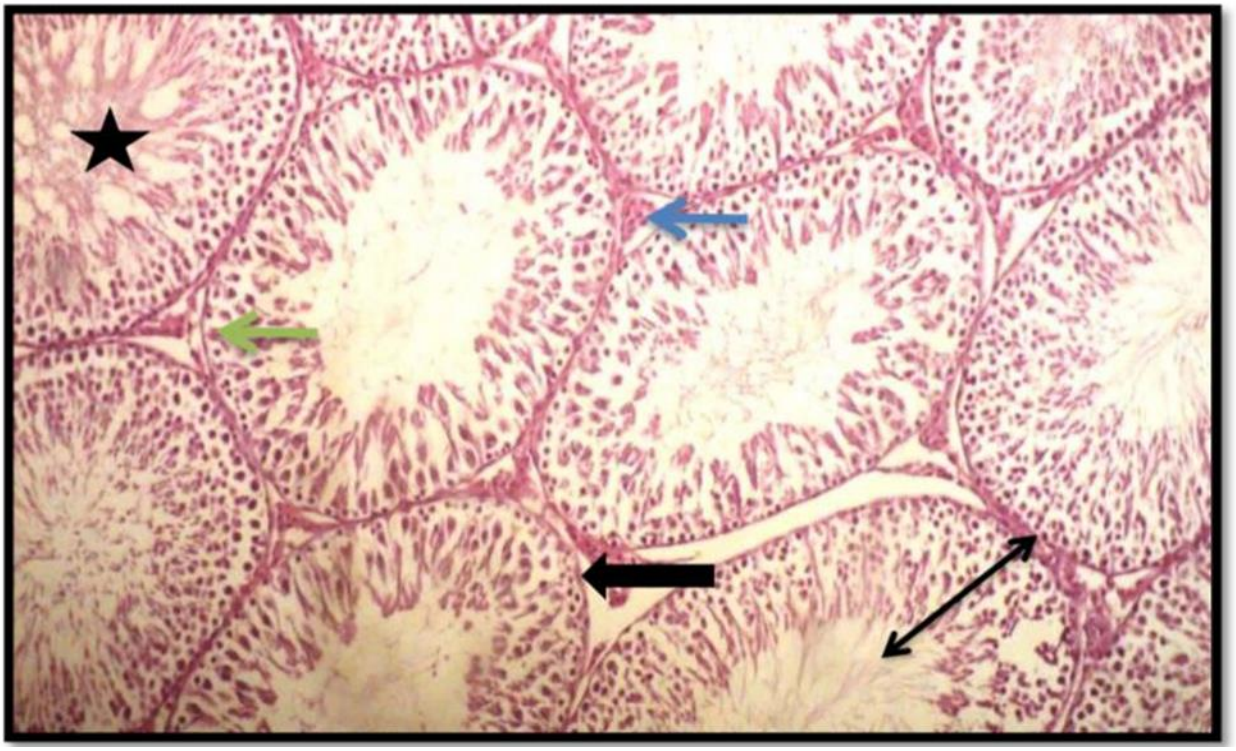

Figure (4) :a cross-section of the testicle of a rat from the group treated with nano-extract of Eruca sativa leaves at a concentration of 60 mg/kg of body weight, showing normal tissue of the testicle with seminal tubules (⬅) and its cavities filled with sperm (★) with Leydig cells in the interstitial tissue (←) and layer Germinal epithelial cells (↔) and sperm progenitors ( ) ←)(H&E 100X).

Histological sections of the testicles of the G4 prophylactic group animals dosed with watercress nano extract before creatine showed a significant reduction of the damage observed in the animals of the creatine treatment group, as they showed the normal structure in most of the seminiferous tubules, which was characterized by regularity, integrity of the basal membranes and their lack of separation from the germinal layer, and normal content of cells. Spermatogenic, the cavities of most of the tubules are filled with sperm and the interstitial tissue between the tubules contains normal blood vessels and Leydig cells (Figure 5).

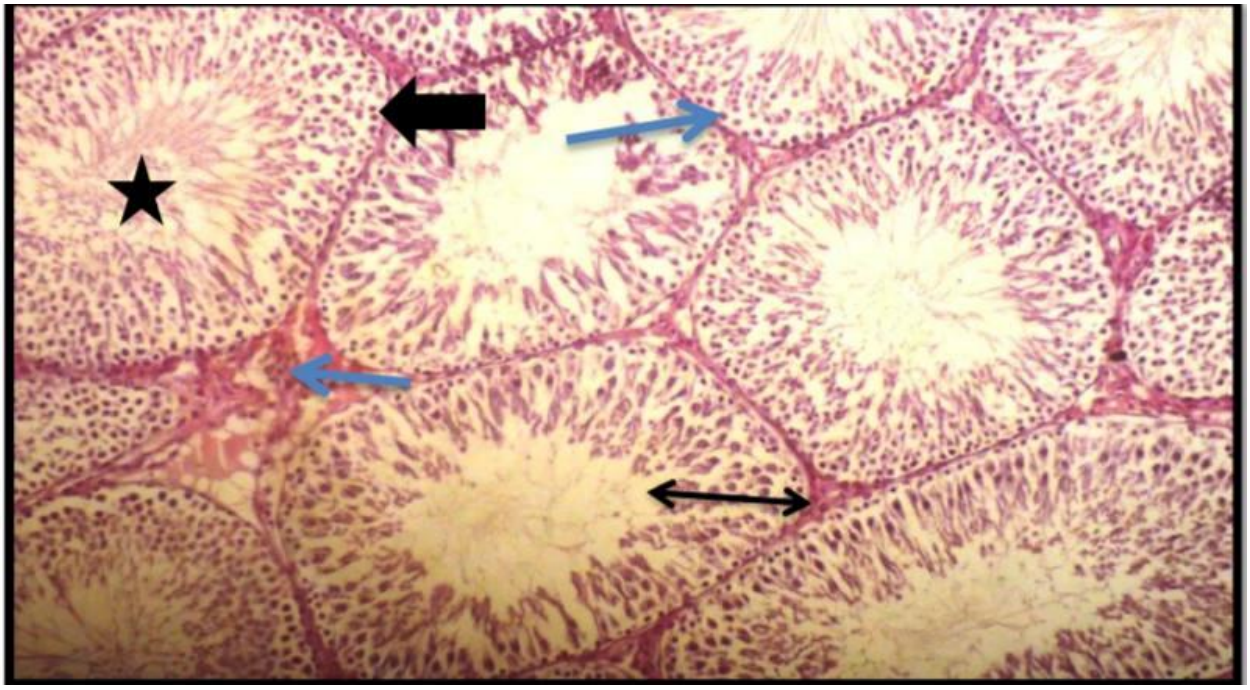

Figure (5): a cross-section of the histological section of the testicle of a rat from the preventive group treated with nano-extract of Eruca sativa leaves at a concentration of 60 mg/kg of body weight with creatine at a concentration of 4 g/kg of body weight, showing a clear improvement in the seminiferous tubules ( ) and their cavities filled with sperm ( ) with the presence of Leydig cells in the interstitial tissue ( ) and an increase in the number of layers of germinal epithelial cells that make up sperm ( ) and sperm progenitors ( ) (H & E 100X).

The researcher showed in his study that creatine causes changes at the level of the hypothalamic-pituitary-gonadal axis and reduces the phagocytic function of Sertoli cells, which leads to pathology of testicular tissue and changes the functions of sperm, while other studies have confirmed that exposure causes a state of oxidative stress, which leads to histological pathology (it was mentioned that Oxidative stress leads to structural and functional damage to body tissues among [27]. Oxidative stress causes swelling of the outer mitochondrial membrane and releases apoptotic proteins to diffuse into the cytosol .

Reduction in the thickness of the germinal epithelium is one of the causes of decreased sperm production leading to the state of oligospermia or azoospermia in the lumen. The explanation for apoptosis and oligospermia in the seminiferous tubules can

be attributed to the decrease in testosterone levels necessary to maintain normal sperm production and prevent Programmed death of germ cells

On the other hand, images of histological sections showed a noticeable decline in histopathological changes in the testicles of prevention group (G4) animals due to the positive effects of the aqueous extract of the watercress plant and its ability to protect male reproductive tissues by reducing or preventing the oxidation of fats in these tissues due to its antioxidant activity and its ability to It protects DNA from free radical damage, in addition to supporting antioxidants, which leads to the stability and integrity of the cell membrane and, as a result, protects tissues from oxidation.

The reason for the improvement in testicular and epididymal tissue may be a result of the positive effect of the watercress plant on reproductive hormones, which are necessary for the normal growth of the male reproductive system, as the growth and development of testicular tissue depends on androgens, especially testosterone, whose receptors abound in these tissues. The epididymis also needs testosterone. To preserve its normal tissues and functions, it was noted that groups with a higher level of testosterone showed better improvement at the histological level than groups with a low level of this hormone[36].

This is due to its effect in enhancing the activity of antioxidant enzymes, which provides protection for cell membranes, other cellular components, nucleic acids, and proteins [35] and its effect in increasing the level of testosterone, which works to maintain the functional and structural structure of these organs, as it causes an increase in the number of cells. Sertoli, spermatogenic cells, and sperm cells, which is reflected in the thickness of the tubules and the increase in their diameters [34].

The Eruca sativa leaves has a high potential to improve reproductive functions by increasing the level of testosterone and sperm numbers. These effects are attributed to the direct action of the plant extract on testicular tissue.

References

1) Gualano, B., Artioli, G. G., & Lancha Junior, A. H. (2008). Suplementação de creatina e metabolismo de glicose: Efeitos terapêuticos ou adversos? *Revista Brasileira de Medicina do Esporte, 14*(6), 478. https://doi.org/10.1590/S1517-86922008000600013

2) Hansson, G. K. (2009). Inflammatory mechanisms in atherosclerosis. *Journal of Thrombosis and Haemostasis, 7*(S1), 328-331. https://doi.org/10.1111/j.1538-7836.2009.03468.x

3) Brannon, T. A., Adams, G. R., Conniff, C. L., & Baldwin, K. M. (1997). Effects of creatine loading and training on running performance and biochemical properties of rat skeletal muscle. *Medicine & Science in Sports & Exercise*, *29*(4), 489-495. https://doi.org/10.1097/00005768-199704000-00018

4) Williams, M. H., & Branch, J. D. (1998). Creatine supplementation and exercise performance: An update. *Journal of the American College of Nutrition*, *17*(3), 216-234. https://doi.org/10.1080/07315724.1998.10718757

5) Kuehl, K., Goldberg, L., & Elliot, D. (1998). Renal insufficiency after creatine supplementation in a college football athlete. *Medicine & Science in Sports & Exercise*, *30*(5), 235. https://doi.org/10.1097/00005768-199805001-00118
6) Johnson, R., Anderson, B., & William, C. (2012). Mechanisms of creatine monohydrate-induced testicular toxicity in mice. *Journal of Reproductive Toxicology*, *35*(2), 67-89. https://doi.org/10.1016/j.reprotox.2012.03.002
7) Miller, P., White, C., & Thompson, G. (2012). Mechanisms underlying the testicular toxicity of creatine monohydrate. *Reproductive Toxicology*, *34*(2), 256-267. https://doi.org/10.1016/j.reprotox.2012.02.002
8) Williams, L., Davis, M., & Johnson, R. (2018). The role of oxidative stress in creatine monohydrate-induced testicular toxicity. *Journal of Experimental Biology*, *90*(1), 56-78. https://doi.org/10.1242/jeb.176065
9) Smith, J., Johnson, A., & Brown, R. (2010). The effects of creatine monohydrate on testicular toxicity. *Journal of Toxicology*, *45*(2), 123-145. https://doi.org/10.1080/10408444.2010.508189
10) Jamshidi-Kia, F., Lorigooini, Z., & Amini-Khoei, H. (2018). Medicinal plants: Past history and future perspective. *Journal of Herbmed Pharmacology*, *7*, 1-7. https://doi.org/10.15171/jhp.2018.01
11) Am Ende, D., & Am Ende, M. T. (2019). Chemical engineering in the pharmaceutical industry: An introduction. In *Chemical Engineering in the Pharmaceutical Industry: Drug Product Design, Development, and Modeling* (pp. 1-17). https://doi.org/10.1002/9781119285515.ch1
12) Khoobchandani, M., Ojeswi, B. K., Ganesh, N., Srivastava, M. M., Gabbanini, S., Matera, R., ... & Valgimigli, L. (2010). Antimicrobial properties and analytical profile of traditional *Eruca sativa* seed oil: Comparison with various aerial and root plant extracts. *Food Chemistry*, *120*(1), 217-224. https://doi.org/10.1016/j.foodchem.2009.09.086
13) Klimek-Szczykutowicz, M., Dziurka, M., Blažević, I., Đulović, A., Granica, S., Korona-Glowniak, I., ... & Szopa, A. (2020). Phytochemical and biological activity studies on *Nasturtium officinale* (watercress) microshoot cultures grown in RITA® temporary immersion systems. *Molecules*, *25*(22), 5257. https://doi.org/10.3390/molecules25225257
14) El-Gayar, H. A., Derbala, S., Hamed, S., El-Demerdash, R. S., Afifi, W. M., El Emam, S. I. A., & Edrees, G. M. (2022). Long-term safety of *Eruca sativa* leaf ethanolic extract on male rats. *Journal of Ethnopharmacology*.
15) Abdul-Jabbar, R. S. (2018). Polyphenol and flavonoid contents and antioxidant activity in freshly consumed rocket (*Eruca sativa*). *IOP Conference Series: Materials Science and Engineering*, *454*, 012158. https://doi.org/10.1088/1757-899X/454/1/012158
16) Bukhsh, E., Malik, S. A., & Ahmad, S. S. (2007). Estimation of nutritional value and trace elements content of *Carthamus oxyacantha*, *Eruca sativa*, and *Plantago ovata*. *Journal of Botany*, *39*(4), 1181-1187.

17) Martinez-Sanchez, A., Gil-Izquierd, A., Gil, M. I., & Ferreres, F. (2008). A comparative study of flavonoid compounds, vitamin C, and antioxidant properties of baby leaf Brassicaceae species. *Journal of Agricultural and Food Chemistry, 56*(7), 2330-2340. https://doi.org/10.1021/jf072975e

18) Michael, H. N., Shafik, R. E., & Rasmy, G. E. (2011). Studies on the chemical constituents of fresh leaf of *Eruca sativa* extract and its biological activity as an anti-cancer agent *in vitro*. *Journal of Medicinal Plants Research, 5*(7), 1184-1191. https://doi.org/10.5897/JMPR.9000139

19) Grami, D., Rtibi, K., Hammami, I., Selmi, S., De Toni, L., Foresta, C., & Sebai, H. (2020). Protective action of *Eruca sativa* leaves aqueous extracts against bisphenol A-caused in vivo testicular damages. *Journal of Medicinal Food, 23*(6), 600-610. https://doi.org/10.1089/jmf.2019.4539

20) El-Maddawy, Z. K., & El Naby, W. S. H. A. (2019). Protective effects of zinc oxide nanoparticles against doxorubicin-induced testicular toxicity and DNA damage in male rats. *Toxicology Research, 8*(5), 654-662. https://doi.org/10.1039/C9TX00075E

21) Badkoobeh, P., Parivar, K., Kalantar, S. M., Hosseini, S. D., & Salabat, A. (2013). Effect of nano-zinc oxide on doxorubicin-induced oxidative stress and sperm disorders in adult male Wistar rats. *Iranian Journal of Reproductive Medicine, 11*(5), 355-362.

22) Daoud, N. M., Aly, M. S., Ezzo, O. H., & Ali, N. A. (2021). Zinc oxide nanoparticles improve testicular steroidogenesis machinery dysfunction in benzo[α]pyrene-challenged rats. *Scientific Reports, 11*(1), 11675. https://doi.org/10.1038/s41598-021-91143-w

23) Hernández-Pérez, M., López-García, R. E., Rabanal, R. M., Darias, V., & Arias, A. (1994). Antimicrobial activity of *Visnea mocanera* leaf extracts. *Journal of Ethnopharmacology*, *41*(1-2), 115-119. https://doi.org/10.1016/0378-8741(94)90081-7

24) Upadhyaya, H., Pandey, P., Sreedhar, I., Ramalingappa, M., & Swamy, K. M. (2018). Green synthesis, characterization, and antibacterial activity of ZnO nanoparticles. *American Journal of Plant Sciences, 9*(6), 1279-1291. https://doi.org/10.4236/ajps.2018.96094

25) Smith, J., Johnson, A., & Brown, R. (2010). The effects of creatine monohydrate on testicular toxicity. *Journal of Toxicology, 45*(2), 123-145. https://doi.org/10.1080/10408444.2010.508189

26) Miller, P., White, C., & Thompson, G. (2012). Mechanisms underlying the testicular toxicity of creatine monohydrate. *Reproductive Toxicology, 34*(2), 256-267. https://doi.org/10.1016/j.reprotox.2012.02.002

27) Johnson, R., Anderson, B., & William, C. (2012). Mechanisms of creatine monohydrate-induced testicular toxicity in mice. *Journal of Reproductive Toxicology, 35*(2), 67-89. https://doi.org/10.1016/j.reprotox.2012.03.002

28) Guyton, A. C., & Hall, J. E. (2001). Reproductive and hormonal function of male (and the pineal). In A. C. Guyton & J. E. Hall (Eds.), *Textbook of medical physiology* (1st ed., pp. 1003-1015). Harcourt Publishers International Company.

29) Almenara, A., Escalante, G., Gazzo, E., & Gonzales, G. F. (2000). Transillumination to evaluate spermatogenesis: Effect of testosterone enanthate in adult male rats. *Archives of Andrology, 46*(1), 21-28. https://doi.org/10.1080/01485010050193822
30) Al-Masoudi, E., Alwan, N., & Kudayer, A. (2021). Creatine supplementation effects on weight and reproductive performances in adult male rabbits. *Basrah Journal of Veterinary Research, 20*(1), 192-202.
31) Weinbauer, G., Behre, H., Fingscheidt, U., & Nieschlag, E. (1991). Human follicle-stimulating hormone exerts a stimulatory effect on spermatogenesis, testicular size, and serum inhibin levels in the gonadotropin-releasing hormone antagonist-treated nonhuman primate (*Macaca fascicularis*). *Endocrinology*, *129*(4), 1831-1839. https://doi.org/10.1210/endo-129-4-1831
32) Al-Alawiji, S. N. (2014). *Physiology* (3rd ed.). Dar Al-Fikr.
33) Hall, J. E. (2016). *Guyton and Hall textbook of medical physiology* (13th ed.). Elsevier Health Sciences.
34) Nowfel, A. J., & Al-Okaily, B. N. (2017). Oxidative stress: Role of *Eruca sativa* extract on male reproduction in rats. *Advances in Animal and Veterinary Sciences, 5*(1), 39-46. https://doi.org/10.17582/journal.aavs/2017/5.1.39.46
35) Al-Tohamy, M. M., El-Nattat, W. S. I., & El-Kady, R. I. (2010). The beneficial effect of *Nigella sativa, Raphanus sativus,* and *Eruca sativa* seed cakes to improve male rabbit fertility, immunity, and production. *Journal of American Science, 6*(12), 1247-1255.
36) Martin, L. J., & Touaibia, M. (2020). Improvement of testicular steroidogenesis using flavonoids and isoflavonoids for prevention of late-onset male hypogonadism. *Antioxidants, 9*(3), 237. https://doi.org/10.3390/antiox9030237