A comparative study on the fertility effects of phytochemical compounds isolated from the fruits of *Euterpe Oleracea L.* and *Lycium Barbarum L.* with Tadalafil drug on male rats

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ABSTRACT
This study was implemented to investigate the effect of Euterpe. Oleracea L. anthocyanin (EOA) and L. barbarum polysaccharides (LBP) on male fertility in comparison with tadalafil. Anthrocyanin was obtained from the crude extract of E. oleracea using solid phase extraction (SPE). polysaccharides were isolated from the crude extract of Lycium barbarum L. using chemical methods. Twenty eight adult male rats were divided into four equal groups; First group (T1), was treated with 80 mg/kg (EOA). Second group (T2), was treated with 100 mg/kg (LBP), third group (T3), was treated with 1.8mg/kg Tadalafil drug. The last group (T4) was treated with distilled water and was considered as control group. Results of sperm (count, motility, and viability), testosterone level and fertility index of T1,T2 and T3 groups showed significant increase as compared with (T4). The (T2) was showed a significant decrease in the abnormality percentage and significant increase in testes weight and Gonadosomatic index as compared with (T4). The (T1) showed a significant reduction of the body, testes weight and gonadosomatic index as compared with (T4). The (T3) showed significant increase of the body, testes weight and gonadosomatic index as compared with (T4). Histological results of the testis in (T1) showed the somniferous tubules were filled with spermatid and mature spermatozoa, while (T2) had hyperplasia in few somniferous tubules in the primary spermatocytes and the somniferous tubules lumen was filled with mature spermatozoa, while (T3) revealed a clear congestion of blood vessels and odema in the interstitial tissue. In this study concluded the Polysaccharides of L. barbarum fruits and anthocyanins of E. oleracea fruit can be considered as fertility-facilitating agents and can reduce the sperm deformities.

دراسة تأثير خصوبة المركبات النباتية المعزولة من ثمار الاساي و ثمار توت غوجي ومقارنتها مع دواء التادلافيل على ذكور الفئران

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الخلاصة
اجريت هذه الدراسة لمعرفة تاثير الانثروسيانين المستخلص من ثمار الاساي والسكريات المتعددة المستخلصة من ثمار عنب الذئب (نبات توت غوجي) على خصوبة الذكور بالمقارنة مع عقار تادلافل. تم استخلاص الانثروسيانين بطريقة الطور الصلب (solid phase extraction) في حين تم استخلاص السكريات المتعددة بالطرق الكيميائية. قسمت ثمانية ملغم/كغم . المجموعة الثانية 80بالانثروسيانين وعشرون جرذ الى اربعة مجاميع متساوية. المجموعة الاولى جرعت ملغم/كغم، بينما المجموعة 1.8 تادلافل جرعت بعقار ملغم/كغم. المجموعة الثالثة 100جرعت بالسكريات المتعددة

الاخيرة اعتبرت مجموعة سيطرة وجرعت بالماء المقطر. اظهرت نتائج الحيوانات المنوية ارتفاع ملحوض في العدد دليل الخصوبة للمجاميع المعالجة بالانثوسيانين والسكريات المتعددة والحركة والحيوية وكذلك هرمون التيستيستيرون و تادلافل بالمقارنة مع مجموعة السيطرة، في حين اظهرت المجموعة المعالجة بالسكريات المتعددة انخفاض ملحوظ وعقار و وزن الخصية عند مقارنتها مع مجموعة السيطرة. بينت في التشوهات وارتفاع حاد في دليل كونادوسوماتك وانخفاض في وزن الجسم ووزن الخصية عند كونادوسوماتك المجموعة المعالجة بالانثوسيانين انخفاض في دليل مع تادلافل زيادة عالية في دليل كونادوسوماتك مقارنتها مع مجموعة السيطرة، في حين اعطت المجموعة المعالجة بعقار زيادة في وزن الجسم ووزن الخصية عند المقارنة مع مجموعة السيطرة. اوضحت نتائج التقطيع النسيجي للخصية في النطف الناضجة. في حين اظهرت النبيبات المنوية في المجموعة الثانية فرط المجموعة الاولى امتلاء النيبات المنوية ب التنسج في التكوين النطف الاولي مع امتلاء جوفها بالنطف الناضجة. بينت المجموعة الثالثة احتقان واضح في الاوعية الدموية وتجمع السوائل بين الانسجة . نستنتج من هذه التجربة ان الانثروسيانين المستخلص من *فاكهة الاساي* والسكريات المتعددة المستخلصة من *ثمار غوجي* لها دور في رفع الكفاءة التناسلية وتقليل التشوهات في الحيوانات المنوية.

1. INTRODUCTION

The reproductive system is one of the most important systems in the entire body. Infertility is a major issue that has been thought to be one of the woman's problem. However, it turned out about one out of every three cases of infertility is due to men alone. The oral prescription medication of Viagra® is effective but has many side effects (1). Oral testosterone can reduce erectile dysfunction, but it is often ineffective and causes liver damage (2). Other drugs such as Yohimbine, papaverine hydrochloride (used under careful medical supervision), phentolamine, and alprostadil (Caverject®) cause the widen blood vessels. In addition, these medications are quiet expensive in the developing countries. Therefore, medicinal plants have been used in the treatment of infertility with a lower coast and a reduced amount of side effects. However, this ethnobotanical indigenous knowledge has not been earlier documented and scientifically validated for efficacy and safety (3). *L. barbarum* (Guji berry) a famous Chinese medicinal herb, has a long history of use as a traditional remedy for male infertility. Polysaccharides are the most important functional constituent in L. barbarum fruits. Polysaccharides improved the copulatory performance and reproductive function of hemicastrated male (4).

E. oleracea (Acai berry) is an extremely effective fruit, filled with antioxidants, minerals, omega-6-9 fatty acids, B vitamins, fiber and protein. Improving blood circulation is an important factor for better sex life as it increases libido. This berry improves cardiovascular health thus it has a preventive effect against cardiovascular diseases. This fruit has a high content of anthocyanins, which is found in much smaller amounts in other fruits. Anthocyanins are plant secondary metabolite that plays a key role in responses related to nutrient availability and in increasing male fertility (5). The aim of this study is to explore the fertility effects of the phytochemical compounds polysaccharides and anthocyanins isolated from *L. barbarum* and *E. oleracea* respectively in comparison with Tadalafil drug on male rats.

As tadalafil inhibits PDE5 activity which causes sexual stimulation that leads to an erection causes the production and release of nitric oxide in the penis. The nitric oxide causes an enzyme guanylate cyclase to produce cyclic guanosine monophosphate (cGMP). It is cGMP that is primarily responsible for increasing and decreasing the size of blood vessels carrying blood from the penis, respectively, and causing an erection. In addition, it is devoid of deleterious effects on semen volume, concentration, sperm membrane integrity or sperm penetration assay (6). This study was aimed to investigate the effect of the most important phytochemical compounds isolated from the fruits of *E. oleracea* and *L. barbarum* on fertility and compare them with Tadalafil drug.

2. MATERIAL AND METHODS

Extraction and purification of L. barbarum polysaccharides (LBP):

Dried fruits were purchased from natura hustopece- EU. Fruits were grinded into fine powder. Then, the sample was refluxed to remove lipids with chloroform: methanol solvent (2:1) (v/v). After filtering, the residue was air-dried, and then refluxed again with 80% ethanol at 80 °C to remove oligosaccharides. The residues were extracted four times in boiled water then filtered. The filtrate was concentrated by a rotavap, and then precipitated using 95% ethanol, 100% ethanol and acetone, respectively. After filtering and centrifuging, the precipitate was collected and vacuum-dried. The dried L. barbarum polysaccharides (LBP) obtained were stored in a refrigerator for the in vivo study (7).

Extraction of *E. oleracea* anthocyanin (EOA):

Dried powder of *Euterpe oleracea* was obtained from ISWARI/ CZ-BIO-001-EU. The powdered fruits were frozen with liquid nitrogen and powdered. The use of liquid nitrogen minimizes anthocyanin degradation by lowering the temperature and providing a nitrogen environment. The fine powder maximizes pigment recoveries due to its high surface area and favors disruption of cellular compartments, later on from the crushed material anthocyanin was extracted by following Methanol and Acetone-chloroform extraction method (8)

Later, the powder was extracted in acid water (distilled water containing 0.01 M HCL) overnight with shaking. The anthocyanin-rich extract was concentrated to dryness in a rotavap. The anthocyanin-rich extract was dissolved in 50 ml of distilled water for anthocyanin purification (9).

Isolation and purification of *E. oleracea* anthocyanin using solid phase extraction (SPE)

Five gram C18 cartridge (Sigma Aldrich) was washed with methanol, the cartridge was washed with acidified deionized distilled water to remove the methanol. 25 ml of the anthocyanin-rich extract was forced through the cartridge and then washed with acidified water to remove sugar and other polar compounds in the extract. Anthocyanin component was eluted with acidified ethanol (0.01% HCl) and collected in a 150 ml flask. The ethanol was totally removed in a rotavap. The concentrated extract was redissolved in acidified water. The final extract was pinkish in color and was stored at -4°C in a refrigerator for the rest of the study. (9 and 10), (Thin Layer chromatography was conducted using an aluminum plate coated by silica gel as stationary phase and n-Butanol: Acetic Acid: Water (4:1:5) as mobile phase (11).

Experimental Animals:

Twenty eight adult male rats weighting 400g were obtained from the animal house of the college of pharmacy/ University of Baghdad. Animals were placed in cages subjected to constant environmental conditions. Standard rodent diet (commercial feed pellets) and Tap water were freely available.

Determining concentrations of (LBP) and (EOA) used in this study:

A pilot study was conducted under the same circumstances for the main experiment in order to determine the appropriate dose of the chosen phytochemical compounds. Therefore, forty adult male rats weighted between 300-400kg were divided into eight subgroups and treated with a dose of (10, 50, 100, 200) mg/kg for **(LBP)** and (20, 40, 80, 160) mg/kg

(EOA). Treatment was given daily for a month by G- tube. The dose of 100mg/kg for **(LBP)** and the dose of 80 mg/kg for **(EOA)** illustrated the best results and were chosen for the main in *vivo* experiment.

The main in *vivo* experiment:

Twenty eight adult male rats were divided equally into four groups. Treatment was administrated daily by G-tube for a month:

First group (T1): Was treated with a dose of 80 mg/kg B.W of **(EOA)**.
Second group (T2): Was treated with a dose of 100 mg/kg B.W of **(LBP)**.
Third group (T3): Was treated with a dose of 1.8mg/kg B.W of Tadalafil drug.
Fourth group (T4): Was considered as control and treated with distilled water only.

Parameters used in this experiment:

Fertility test (mating test)*:* This index was determinate according to (12) as each male rat was caged separately with 2 females to confirm fertility for 6 days. The presence of sperms in the vaginal smears examined after one day indicated that the females had mated to the exacting male and the day of mating was taken to be first day of pregnancy. Fertility test was considered positive when implantation sites were present.

Body and testes weight: Body weight of the rats was measured at the first day using a mechanical balance before dosage and every week during treatment. In addition, all testis were removed and weighed after animals were subjected to euthanasia.

Concentration of testosterone*:*

After evaluation the rats fertility by mating test, animals were subjected to euthanasia. Then blood was collected by cardiac puncture, and serum was separated. Concentration of testosterone was determined by Radio-immunoassay (RIA) (13).

Semen Collection and Analysis:

All testis were removed along with the epididymides. Afterward, the caudal epididymides were separated from the testis. Then, epididymides were blotted with filter papers and mangled for semen collection.

Epididymal contents of the treated rats were obtained after cutting the tail of epididymis***;*** 100 mg of caudal epididymis was minced with 5 ml of 0.9% NaCl and mixed well. Then, one drop of this mix was applied to hemocytometer chamber under cover slip. Quantitative motility percentage was determined by counting motile and immotile spermatozoa per unit area. While, quantitative viability percentage was determined by counting viable and unviable spermatozoa per unit area. Moreover, sperm count was determined according to (14). Sperm morphology was done by adding two drops of warm Eosin/Nigrosin stain to the semen on a pre-warmed slide. After that, a uniform smear was air-dried. Then, the stained slide was immediately examined under the microscope using x400 magnification (15).

GSI (Gonadosomatic index): This index is determined according to (16) as it indicates testis weight/body weight ratio x 100.

Histological sectioning of testes tissue: Specimens of testes tissue were taken and kept in 10% formalin for fixation. Then processed routinely in histokinette. After that, it was

embedded in paraffin wax and stained with haematoxylin and eosin. Finally, examined under a light microscope (17).

Statistical analysis: SAS (18) was used for statistical analysis to study the effect of different treatments. Also, the mean was compared with less significant LSDs.

3. RESULTS:

TLC for (EOA): The extracted and isolated anthocyanin *from E.oleracea* fruits gave one spot using thin layer chromatography technique .The Rf value for TLC was 0.18

Pilot study: Motility percentage and sperm count in groups treated with (EOA) and (LBP) showed a gradual significant increase ($P< 0.05$) in contrast with the control group until it reached 80 mg/kg for (EOA) and 100 mg/kg for (LBP) treated as in tables (1-2)

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Table (1): Illustrates the Motility Percentage and Sperm Count ($x\ 10^6$) for Different Doses of (EOA).

Doses of (EOA) mg/kg	Motility%	Sperms count (x 10^6)
control	80 ±1.3 A	40.16 ± 1.33 A
20	85 ± 1.5 B	60.41 ± 2.52 B
40	88 ± 1.9 C	77.60 ± 2.91 C
80	92± 1.6 D	82.79 ±3.01 D
160	91 ± 1.1 D	82.08.11 ±2.09 D

*Data taken as mean ± SE ** Different capital letters mean significant difference ($P<0.05$) between column numbers.

Table (2): Illustrates the Motility Percentage and Sperm Count ($x\ 10^6$) for Different Doses of (LBP).

Doses of (LBP) mg/kg	Motility%	Sperms count (x 10^6)
control	80 ±1.3 A	40.16 ± 1.33 A
10	87 ± 1.6 B	70.12 ±2.41 B
50	90±1.7 C	80.53 ± 2.90 C
100	96± 1.5 D	92.37 ±2.10 D
200	97.6 ± 1.0 D	91.33 ±2.10 D

*****Data taken as mean ± SE ****** Different capital letters mean significant difference ($P<0.05$) between raw numbers.

Semen analysis and fertility index: Sperm count, motility and viability percentage of (T1-T2-T3) showed a significant increase ($P< 0.05$) when compared with (T4). While, (T2) showed a noticeable increase ($P< 0.05$) when compared with the rest of the groups. The abnormality percentage of (T2) illustrated a clear decrease ($P< 0.05$) when compared with (T1-T3-T4). While (T3) demonstrated absolutely no difference ($P< 0.05$) when compared

with (T4). Fertility index of (T1-T2-T3) showed significant increase (P< 0.05) when compared with (T4) as in table 3.

Table (3): The Effect of (EOA) 80 mg/kg B.W, (LBP) 100 mg/kg B.W., and Tadalafil drug 1.8mg/kg on Motility%, Viability%, Abnormality %, Sperm Count (x 10^6) and Fertility Index (%).

Groups / Parameters	T1	T2	T3	T4
Motility%	90 ± 1.4 a	95 ± 1.8 b	90 ± 1.5 a	79 ± 1.1 c
Viability %	92 ± 1.9 a	98 ± 2.7 b	91 ± 1.6 a	82 ± 0.9c
Abnormality %	14 ± 3.9 a	9 ± 2.8 b	33 ± 4.3 c	30 ± 4.1 c
Sperm count (x 10^6)	82.44 ±3.21 a	92.80 ± 3.95 b	44.53 ±2.89 c	40.71± 2.97 c
Fertility index	95 ± 2.0 a	98 ± 2.8 b	95 ± 2.1 a	88 ± 1.8 c

*****Data taken as mean ± SE. **Different small letters mean significant difference (P<0.05) between raw numbers.

Body and testis weight: (T1) showed significantly reduction (P< 0.05) in body and testis weight when compared to the control group (T4) and other groups. While (T2) showed a significant decrease (P< 0.05) in body weight and noticable increase (P< 0.05)in testis weight when compared with the control group. Tadalafil treated group (T3) showed a clear increase (P< 0.05) in body and testis weight when compared with (T4) as in table 4.

Table (4): The effect of (EOA) 80 mg/kg B.W, (LBP) 100 mg/kg B.W. and Tadalafil Drug 1.8mg/kg on Serum Testosterone Level (mg/ml), Gonadosomatic Index (%), Body Weight (g) and Testis Weight (g).

*****Data taken as mean ± SE ****** Different capital letters mean significant difference ($p<0.05$) between raw numbers

Gonadosomatic Index and testosterone level: (T1) demonstrated a sharp decrease ($p<0.05$) when compared with the control group (T4), while (LBP) treated group and Tadalafil treated group (T3), illustrated a significant increase ($P\leq 0.05$) when compared with the control group (T4). Moreover, testosterone levels in groups treated with (EOA), (LBP) and Tadalafil showed a clear raise ($P\leq 0.05$) in contrast with the control group as in table 4.

Histological changes:

Histological section of the testis of the experimental animals treated with (EOA) had their somniferous tubules filled with spermatid and mature spermatozoa (figure1) , the section in testes of rat treated with (LBP) had hyperplasia in few somniferous tubules in the

Groups / Parameters	T1	T2	T4	T5
Body weight	350.93 ± 7.90 a	361.03 ± 8.61 b	512.06 ± 7.99 c	400.05 ± 6.46 d
Testis weight	1.80 ± 0.32a	3.01 ± 0.37b	2.99 ±0.30b	2.20 ± 0.25c
Gonadosomatic index	0.51 ± 0.12 a	0.83 ±0.16 b	0.58c ±0.10 c	0.54d ±0.14 d
testosterone level	7.5 ± 1.40 a	10.7± 1.81 b	7.9 ±1.99 a	3.8 ± 1.18 c

primary spermatocytes. Other somniferous tubules showed the lumen filled with mature spermatozoa (figure2). While a testis section in rats treated with Tadalafil drug revealed congestion of blood vessels and odema in the interstitial tissue. Moreover, there were degenerative changes in the spermatocytes and spermatids (figure3) in contrast with the normal section in rat testis.

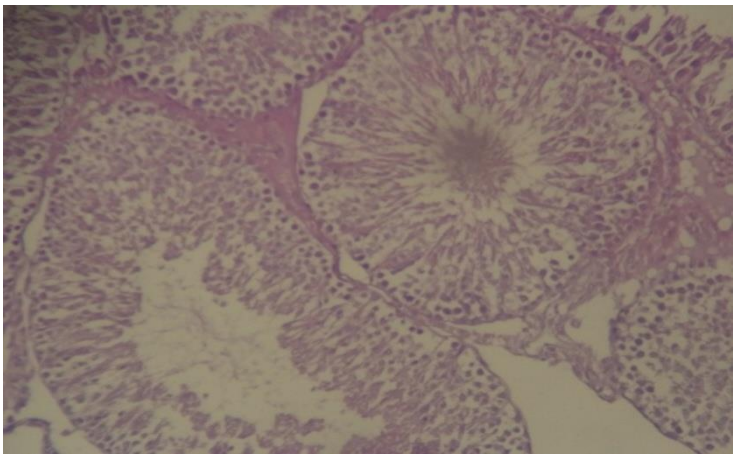

Figure (1): Histological section in testis of rat treated with (EOA) (80 mg/kg) B.W for a month, showed the somniferous tubules were filled with spermatid and mature spermatozoa and the lumen filled with spermatocytes (H&E. X400).

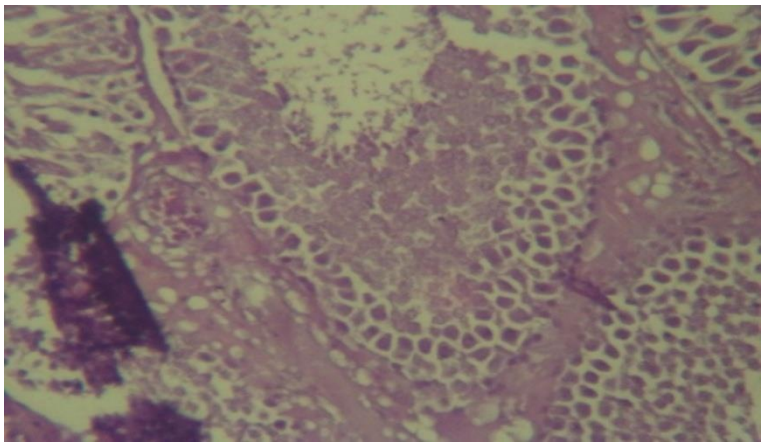

Figure (2): Histological section in testis of rat treated with (LBP) (100 mg/kg) B.W for a month, showed hyperplasia of few somniferous tubules in the primary spermatocytes, while other somniferous tubules showed the lumen filled with mature spermatozoa (H&E. X400).

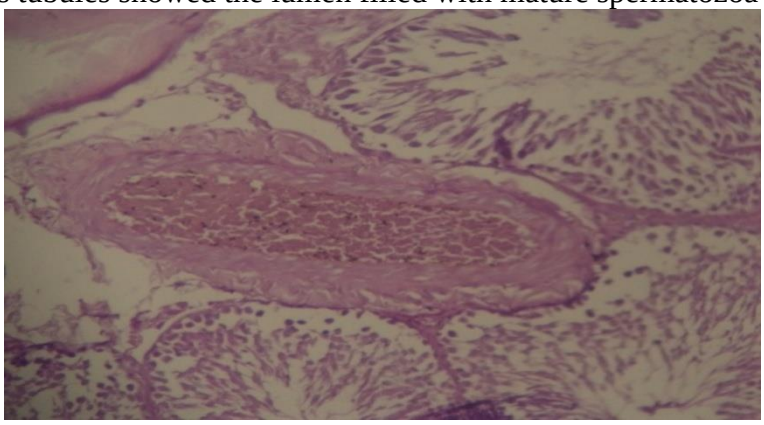

Figure (3): Histological section in testes of rat treated with Tadalafil drug (1.8 mg/kg) B.W for 30 days , shows congestion of blood vessels and odema in the interstitial tissue. Also, some somniferous tubules had pink material in there lumen. Moreover, there were degenerative changes in the spermatocytes and spermatids (H&E. X400).

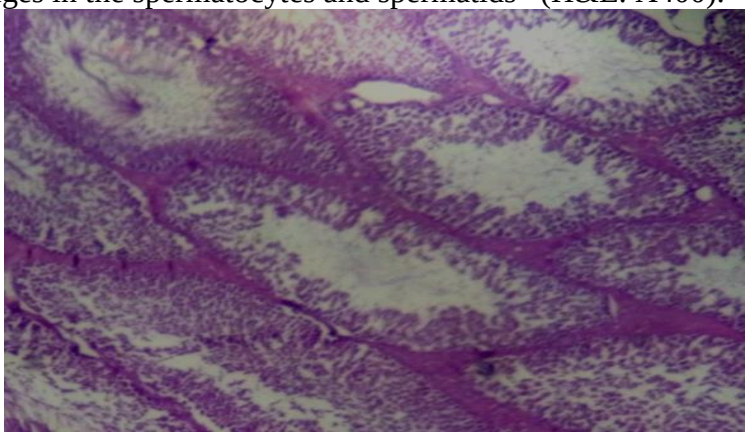

Figure (4): Histological section of testes in rat treated with distilled water for 30 days showed normal tissue (H&E. X400).

4. DISCUSSION

The increase in the sperm count, motility, viability percentage of (EOA) treated groups could indicate that the phytochemical anthocyanin from *E. oleracea* has the ability to decrease the oxidative stress of testis and enhances nitric oxide preservation and bioavailability, which led to increase the fertility. Moreover, animals treated with (EOA)

illustrated a decreased number of abnormal sperms and increased sperm count, this outcome agreed with results reported by (4). While better results of sperm count, motility and viability percentage in rats treated with (LBP), which might be attributed that polysaccharides from *L. barbarum* improved the copulatory performance and the reproductive function in animals by two mechanism: firstly, through regulation of the secretion of sexual hormones including the gonadotropin-like which promotes the hypophysis secretion of gonadal hormone and regulate the hypothalamic-pituitary-gonadal axis in a multiple manner. Secondly, (LBP) may increase the performance of spermatogenic cells through supporting the process of lipid peroxidation and other peroxide radicals on DNA. Also, increased hormone levels and raised accessory sexual organ weights and Gonadosomatic. That lead to improve the sperm count, quantity, quality, improve fertility, decrease abnormalities of sperms, increase weight and coefficients of testis. These outcomes came in agreement with the finding of Zhang (19).

Furthermore, the increase in motility and viability percentage of Tadalafil drug treated group could be regarded to a positive effect of PDE5 inhibitors on sperm motility both in *vivo* and in *vitro* because it has a role in the reduction of ejaculation-associated stress, resulting in an ejaculation with higher sexual satisfaction and a subsequent increased number of spermatozoa in the semen providing an ideal environment for sperm motility and may explain the higher sperm motility profiles in semen samples. Therefore, this result agreed with results reported by (20). In addition, the inhibition of sperm PDE5 has been shown to increase cAMP and cGMP which lead to the raise in semen volume, concentration, sperm membrane integrity or sperm penetration assay. There are studies that agree with these result recorded by (6). Additionally, Tadalafil drug had a role in capacitation and a debated one concerning acrosome reaction and in increase nitric oxide that increase fertility index , this result agreed with the finding of Nikki (21).

Also, the drop off of Body, testis weight and Gonadosomatic index in (EOA) treated group could be due to the inhibitory effects of anthocyanins on body fat accumulation, this effect was probably due to suppression of lipid synthesis in the liver and in white adipose tissue. This result agreed with an upshot reported by (22). while the reduction of body weight in (LBP) treated group could be related to the increased metabolic rate and decreased waist circumference. Therefore, further studies are necessary to confirm the effect on decrease cortisol and related hormone levels, as well as to clarify the interaction with other intrinsic factors such as thyroid hormone levels, which also play important roles in a metabolic system and reduced serum total cholesterol and low density lipoprotein (23).

The increase of body and testes weight and Gonadosomatic index of Tadalafil drug treated group might attributed to inhibition of phosphodiestras (PDE5) and increase nitric oxide led to increase growth hormone, acting as appetizer then increase food and water intake (24).

More to the point, the increase in testosterone level in (EOA) treated group can be due to the protective effect of anthocyanins against stress-induced oxidative damage, as indicated by decreased lipid peroxidation level, increased oxygen radical absorbance capacity (ORAC) and glutathione (GSH) content, along with elevated superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities. This lead to the elevation in testosterone level (25). Even though, the rise in testosterone level in (LBP) treated groups might be attributed to the effect of polysaccharides in the reduction of cortisol hormone in the body and by its antioxidant effect. This result came in agreement with the end result reported by Lee (26).

Furthermore, the increase in testosterone levels in the group treated with Tadalafil drug could be due to the effect of Tadalafil in increasing testosterone: estrogen ratio mainly

related to reduction of estrogen levels lead to the effect on aromatase pathway by interfering with phosphodiesterase isozymes. This result agreed with (27).

Also, results of the histological section of (EOA) treated group regarded to the effect of anthocyanin in removing superoxide, singlet oxygen, peroxide, hydrogen peroxide, and the hydroxyl radical. It also effected in stabilizing or inactivating free radicals and preventing cellular oxidative stress. In addition, anthocyanin may be effective in preventing rapid cell death by apoptosis induced by oxidative stress, which is important for the protection of normal cells from oxidative stress. Anthocyanin accelerates the antioxidant response element-regulated phase II enzyme activity (28).

Moreover, results of the histological section of (LBP) treated group might be regarded to its beneficial effects on the reproductive system at cellular levels and protected the spermatogenic cells from apoptosis and preserved the structural integrity of the seminiferous epithelium by its effect as antioxidant and regulated the hypothalamic-pituitary-gonadal axis. This had lead to the protection of gametes and enhancement of sexual behavior. This result agreed with results reported by Lee (26).

Finally, result of the histological section of Tadalafil drug treated group could be due to Tadalafil raised the concentration of nitric oxide in the tissue and also had the ability to effect on the spermatozoal membrane and revealed congestion of blood vessels by increase blood volume, which gave a pink material in the lumen of somniferous tubules and this result explain the mild abnormalities occurred in sperms. The same result reported by Dimitriadis (29).

CONCLUSION

This study provides evidence for the use of (LBP) and (EOA) as an aphrodisiac and fertility-enhancing agents. Moreover, they improved the quality and quantity of sperms and reduced sperm deformation.

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