The Protective Effect of Quercetin and Hesperidin on Etoposide Induced Toxicity in Male Rats Testicular

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Abstract :

This study aimed to evaluate the synergistic protective role of Quercetin (QE) and hesperidin (HES) alone or in combination against Etoposide-induced in male rats. as used (50) male mature , these were divided into five groups included each group 10 animals. Control group (C) : was administered orally Distilled water, The first treatment group (T1): was given orally Etoposide (20) mg / kg bw ,   Second treatment group (T2) : was given Etoposide (20) mg / kg , then given Quercetin 20 mg / kg,, Three treatment group (T3): was given Etoposide (20) mg / kg then Hesperidin at dose 25 mg / kg bw,   Four treatment group (T4): was given Etoposide and Quercetin   and Hesperidin at same doses previous . The animals were sacrificed after the treatment period. There was statistically significant (p<0.05) Etoposide caused significant a decrease (P<0.05) in genes expression (LH subunit , LHr , CYP11A1) , reduction in plasma LH , FSH and testosterone levels and decrease (P<0.05) in sperm count and also a decrease (P<0.05) in sperm activity of the percentage of viable sperm and an increase in abnormal sperms , reduced (P<0.05) in Fertility rate, number and weight of birth compared with control group and other treated group . Furthermore, Led treatment QE and / or HES with Etoposide -treated group improved the harmful effects of Etoposide by return the parameters rates to normal values rather and was more significant in the synergy (T4) as compared with control group .

In conclusion, Index study revealed Hesperidin and Quercetin both showed synergistic potential more than if they were separate by improving oxidative damage Etoposide – Induced on reproductive system parameters in male rats .

Key words : Protective, Hesperidin , Quercetin, Etoposide , Testicular, Toxicity , Male Rat

INTRODUCTION :

Flavonoids are naturally occurring compounds found in plants that are considered as secondary metabolites and have important medical properties such as antioxidants that inhibit lipid peroxidation in biological membranes (1). these flavonoids may serve as potential chemotherapeutic agents . Hesperidin is a flavanone glycoside, abundantly found in lemon and oranges. The peel and membranous part of these fruits have the highest hesperidin concentration . Hesperidin exhibits various biological and pharmacological properties such as vitamin-like activity and can decrease capillary permeability (vitamin P), leakiness and fragility. It also showed antioxidant, anti-inflammatory, anticarcinogenic ,   anti-bacterial, anti-viral, anti-allergic, and anti-ulcer anticancer, lipid-lowering, antioxidant,   vasoprotective   and antihypertensive and protect against ischemia-reperfusion tissue damage (2) . Citrus flavonoids have also been found to exert synergistic effects with various known chemotherapeutic agents . Quercetin is one of the most frequently studied bio-flavonoids in its class of flavonols. Quercetin is present in high concentrations in fruits and vegetables such as tea, apples, mulberries, onions, potatoes, broccoli, and red wine. It has been shown to have   highly potent antioxidant   and   cyto-protective effects in preventing endothelial apoptosis caused by oxidants (3) .Quercetin is a more potent antioxidant than other antioxidant nutrients, such as vitamin C, vitamin E, and β-carotene, and it can chelate transition metal ions, including iron, thus preventing the iron-catalysed Fenton reaction (4). Treatment with quercetin has been shown to prevent liver damage and suppress overexpression of the inducible form of nitric oxide synthase (iNOS), which is induced   by various inflammatory stimuli (5) .

In general, treatment with anticancer drugs and radiation creates a state of oxidative stress in the body because their side effects, and active oxygen triggers apoptosis via p53 and cytochrome release from mitochondria. Anticancer drugs whose main mechanisms of action involve active oxygen include the anthracyclines , bleomycin, cisplatin and Etoposide . Redox control is also involved in various issues related to anticancer drug therapy . Although the main cause is considered to be the consumption of antioxidants to eliminate the oxidative state, persistent imbalance in the redox state in the body due to anticancer treatment may also be a cause in cases of secondary cancer associated with anticancer drugs or radiotherapy.   With regard to the side effects of anticancer drugs, that tumor cells receive damage as well as normal cells (6) .

Etoposide (VP-16-213), [40-demethylepipodo- phyllotoxin-9-(4,6-O-ethylidene) -b-d - glucopyranoside] , is a semisynthetic derivative of podophyllotoxin, a naturally occurring compound extracted from the roots and rhizomes of the plants, *Podophyllum peltatum* . Etoposide inhibits the topoisomerase II enzyme and/or induces direct DNA breaks, and is used for treatment of a wide range of malignancies (e.g. small cell lung cancer, testicular cancer , acute leukemia, lymphoma) as a single agent or as one of the standard therapeutic regimens (7). The   effects on spermatogenesis of chronic exposure to etoposide are degenerative changes in spermatogonia and early spermatocytes, appearance of large spermatids with multi-nuclei,   and   nuclear   alterations   and   cytoplasmic vacuolation in Sertoli cells (8). Previous reports from our

laboratory demonstrated that exposure to the chemotherapeutic agent Etoposide induced DNA strand breaks in the sperm (9) in addition to affecting fertility by increasing preimplantation and postimplantation loss and abnormal progeny (10 , 7). Consequently, the aims of the current study are to evaluate the therapeutic effects of Quercetin and/or Hesperidin against oxidative stress and Reprotoxicity induced by Etoposide (ETO) in male rats .

MATERIAL AND METHOD:

Used in the experiment 50 rats of the Rattus norvegicus breed. The rats were breeding in the Animal House of the Faculty of Veterinary Medicine / Al-Qadisiyah University. All animals were subjected to similar lab conditions in terms of lighting (12 hours of lighting - 12 hours of darkness) and temperature of 22-25 m . The weights of the animals ranged from one week to 180-200 g. Their ages ranged from 14-16 weeks.

Chemicals :

Etoposide was obtained from NIPON KAY AKU CO LTD (TOKYO 100-0005.JAPAN) , Quercetin and Hesperidin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Their were dissolved in distiller water and stock prepared each week.**.** Etoposide was dissolved in distillated water to achieve the desired concentrations. Fresh solution were prepared weekly and stored in glass containers. Based on the body weight of male rats dose was administered to each one .

Experimental design:

In the experiment, Animals were divided into five groups comprising (10) animals each :

Control Group: served as (normal control) and received distilled water for two months..

T1 (ETO) : (Positive control) is given Etoposide at dose 20mg/kg b.wt daily for 2 month.

T2 (ETO + QE) : was administrated orally Etoposide at same dose (20mg/kg) , then Quercetin 20 mg/kg

T3 (ETO + HES): treated orally with Etoposide at same dose (20mg/kg) , then hesperidin 25 mg/kg

T4 (ETO + QE + HES): was given orally Etoposide , Quercetin, hesperidin , at dose 20 , 20 , 25 mg/kg respectively .

Hormonal assay : Serum concentrations of Testosterone , Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) they were measured by enzyme-linked immune sorbent assay (ELISA) as described in the instructions provided by manufacturer's kits from Monobind Inc .

Sperm analysis : Sperm count was estimated by method described by (11), While **sperm motility** was estimated according to (12), **sperm grade activity** was determined by method (11). The assessment of **sperms viability** (live and dead) was according to the method of (13),While the method of (14) was used to evaluate the abnormal sperm **morphology** .

Fertility test : After 60 days of treatment, five animals (5 rats) leftover from each group were used to measure the fertility index by using 50 female rats (10 females per group).

Tissue specimens :

All animals in the current study were sacrificed at the end of experiment and samples of testis and pituitary glands were taken, made flashing by put them in liquid nitrogen (-196 Cº) and store in deep freezer at -20Cº, to study gene expression for CYP11A1 which responsible for Testosterone hormone production, LH receptor gene and LH beta sub unit gene which responsible for LH production. The used real time PCR primers were designed by NCBI gene Bank data base and primer designed online, these primers were supported from (Bioneer, Korea) company.

Primer		Sequence	Amplicon
CYP11A1	F	GACGCATCAAGCAGCAAAAC	79bp
	R	ATGGACTCAAAGGCAAAGCG	
LH receptor	F	TCACAGACTTCACGTGCATG	70bp
	R	ATAAGGGGCACTTTGAAGGC	
LH subunit	F	AGGACTCAACCAATGACCTGTG	127bp
	R	AAGCCTTTATTGGGAGGGATGG	
GAPDH		AACGACCCCTTCATTGACCTC	82bp
		TGACTGTGCCGTTGAACTTG	

Total RNA were extracted from testis and pituitary tissue of rat by using (TRIzol® reagent kit) and done according to Bioneer company instructions/Korea. The extracted total RNA was assessed and measurement by Nano drop spectrophotometer (THERMO. USA). Two quality controls were performed on extracted RNA. Firstly to determine the concentration of RNA (ng/µL), and secondly the purity of RNA; by reading the absorbance at 260 nm and 280 nm in same Nano drop machine . The extracted total RNA were treated with **DNase I enzyme** to remove the trace amounts of genomic DNA from the eluted total RNA by using (DNase I enzyme kit) and done according to method described by Promega company, USA. DNase-I treatment total RNA samples were used in cDNA synthesis stage by using (AccuPower RocktScript RT PreMix kit) that provided from Bioneer company/Korea, and prepared according to instructions of company. qPCR master mix was prepared by using AccuPower TM Green Star Real-Time PCR kit based SYBER green dye which detection gene amplification in Real-Time PCR system and done according to Bioneer company instructions/Korea. After that, these qPCR master mix above Accopwer Green star qPCR premix standard plate tubes that contain the syber green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, then placed in Miniopticon Real-Time PCR system. After that the qPCR plate was loaded and the following thermocycler protocol in the following table:

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	3 min	**1**
Denaturation	95 °C	20 sec	**45**
Annealing\Extention Detection(scan)	60 °C	30 sec	
Melting	60-95°C	0.5 sec	**1**

The results of qRT-PCR for housekeeping and target genes were analyzed via the relative quantification gene expression level (fold change) ΔCT according to the Livak method (15) Relative quantification method quantity obtained from qRT-PCR experiment must be normalized in such method that the data become biologically significant. In this method one of experimental samples is the calibrator as control sample each of the normalized CT values (target values) is divided by the calibrator normalized target value to produce the relative expression levels, after that the ΔCT method with a reference gene was used as following equations:

Gene	Test (Treatment group)	Cal. (Control group)
Target gene	CT (target, test)	CT (target, cal)
Reference gene	CT (ref, test)	CT (ref, cal)

First; normalize the (CT) of the reference gene to the target gene, for calibrator sample:

ΔCT (control) = CT (ref, control) – CT (target, control)

Second; normalize the CT of the reference gene to the target gene, for the test sample:

ΔCT (Test) = CT (ref, test) – CT (target, test)

ΔΔCT= ΔCT (test) – ΔCT (control)

Fold change = 2-ΔΔCT, Ratio (reference /target) = 2CT (reference) – CT (target). So, the relative expression was divided by the expression value of chosen calibrator for all expression ratio of test sample.

Statistical analysis :

Results were expressed as the mean standard error (M±SD) of the mean (SEM). One-way ANOVA was applied to test for the significance of biochemical data of the different groups Significance is set at (P<0.05).

RESULT :

1) Molecular analysis :

a) Total RNA concentration and purity

RNA concentration and total purity were measured and extracted from experimental animal tissue (Testicular and Pituitary) by the Nanodrop device. The results showed that the total concentration of total RNA concentration in the testis showed the following rates : (389.76 ± 0.58, 326.78 ± 3.14, 381.16 ± 0.42 , 371.18 ± 2.1 , 383.1 ± 0.79) For treatments (C, T1, T2, T3, T4) respectively . While the results of the total concentration of the pituitary total RNA concentration of treatments (C, T1, T2, T3, T4) the following rates (353.4 ±0.9 , 312.8 ± 1.37 , 346.23 ± 0.99 , 341.5 ±0.76 , 351.6 ±0.47) respectively .

The purity of the extracted RNA was estimated with absorbance ranging from (A 260 / A 280) and obtained the testicular mRNA results (1.81± 0.007 , 1.65 ± 0.007 , 1.77 ± 0.008 , 1.77 ± 0.005 , 1.79± 0.002) respectively for treatment (C, T1, T2, T3, T4) , Pituitary total RNA purity was also obtained ((1.75± 0.009 , 1.62± 0.005 , 1.70 ± 0.003 , 1.69± 0.003 , 1.73 ± 0.005) for treatment (C, T1, T2, T3, T4) respectively . (Figure 15 , 16 , 17) .

b) Gene expression of (CYP11A1, LH receptors , LH beta subunit)

The results of the study shown in Figure (1,2,3) a significant decrease (P <0.05) in the gene expression of (CYP11A1, LH receptors , LH beta subunit) in the group treated with Etoposide at a dose (20 mg / kg b.wt) when compared with the control group and with the other groups , While not recorded the results significant difference (P <0.05) in the treatment groups (T2, T3, and T4) compared to the control group as well as with each other .
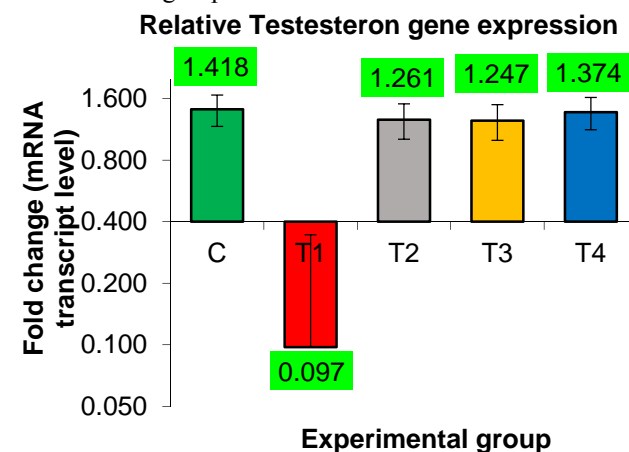

Figure1 : Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on Gene Expression of Cytocrome 11A1 (CYP11A1).

Values are expressed as Mean ± SE for five animals in the group.

- Different small letters are different significant (P<0.05) as compared to respective Control .
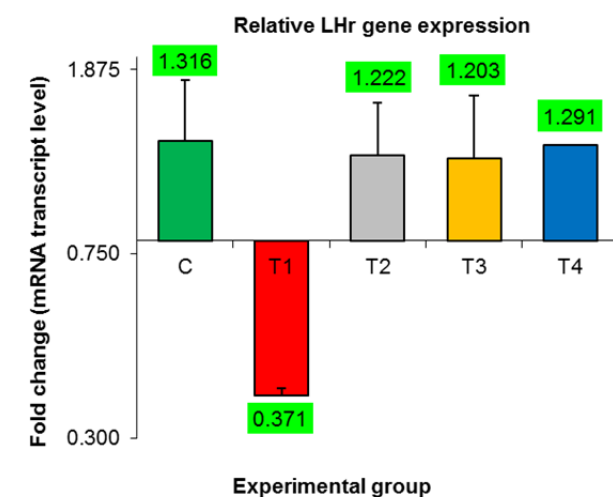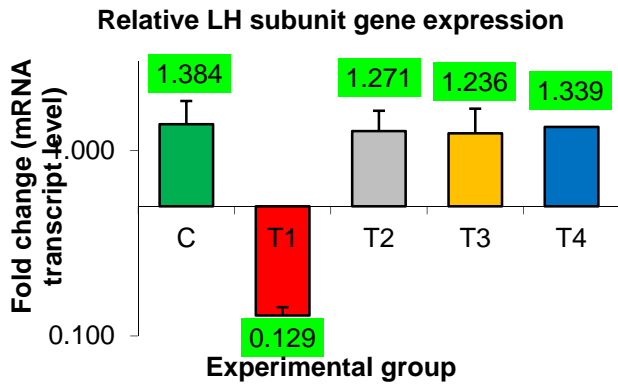

Figure 2: Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on Gene Expression of LH receptor .

Values are expressed as Mean ± SE for five animals in the group.

- Different small letters are different significant (P<0.05) as compared to respective Control .
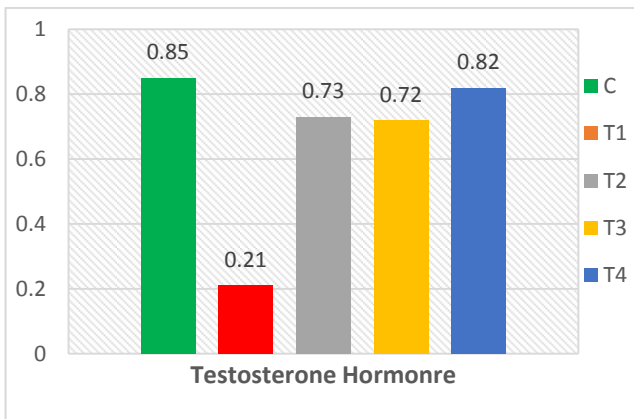
Relative LH subunit gene expression

Figure3 : Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on Gene Expression of LH Beta Subunit .

Values are expressed as Mean ± SE for five animals in the group.
- Different small letters are different significant (P<0.05) as compared to respective Control .

Figure 4: Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on **Testosterone level** .

Values are expressed as Mean ± SE for five animals in the group.
- Different small letters are different significant (P<0.05) as compared to respective Control .

2) Hormonal essay :

The results of the current study in (Figure 4) showed a significant decrease (P <0.05 in the concentration of testosterone hormone in animals treated with Etoposide (T1) compared to control as well as other groups (T2, T3 and T4). The results showed a significant decrease (P <0.05) in the (T3,T2) groups compared with control group and (T4), while the ratio of (T3) and (T2) did not show a significant difference (P> 0.05) . There were no significant differences (P> 0.05) in control group and (T4) when compared with each other.

The results of the statistical analysis shown in (Figure 5) presence a significant decrease (P <0.05) in the concentration of Luteinizing hormone in the treatment group with Etoposide (T1) compared to control group and other groups (T2, T3, T4) .The results showed significant

difference (P <0.05) in the (T2 ,T3) compared to the control rate, while the results did not show a significant difference between T4 and control, while (T2 and T3) were also not significant (P> 0.05) . whereas showed a significant difference (P> 0.05) between (T4) and (T3) but not with (T2).

Results have seen in (Figure 6) a presence significant difference (P <0.05) in the follicle stimulating hormone in the group treated with Etoposide (T1) when compared with the group rates (T2, T3 and T4) and control group. (T2 , T3) compared to the control rate, as compared to the groups mentioned above , while the results showed no significant difference (P> 0.05) in control group and (T4) ratios when compared with each other . Result there was a significant difference (P> 0.05) between (T4) when compared with treatments (T3, T2) .
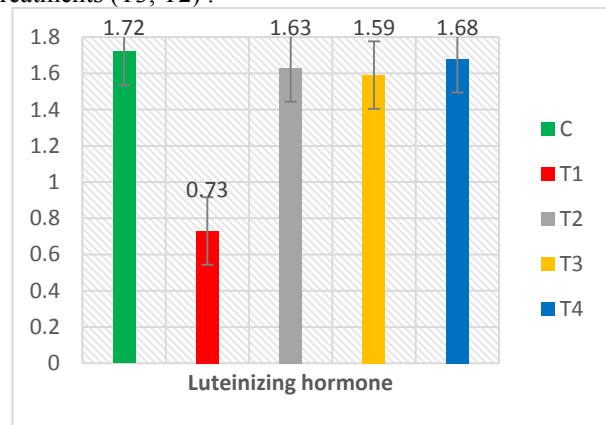

Figure5 : Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on Luteinizing hormone level .

Values are expressed as Mean ± SE for five animals in the group.
- Different small letters are different significant (P<0.05) as compared to respective Control .
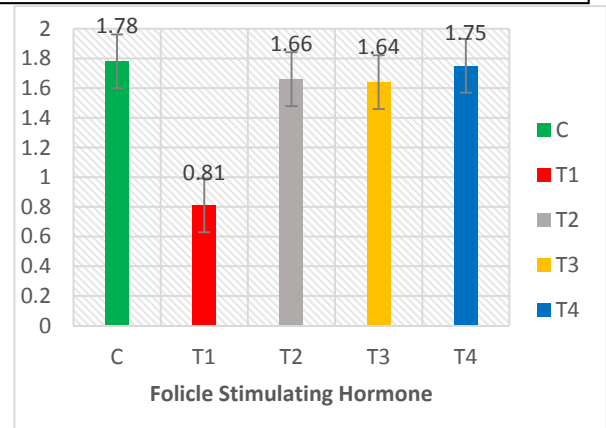

Figure6 : Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on Follicle Stimulating Hormone .

Values are expressed as Mean ± SE for five animals in the group.
- Different small letters are different significant (P<0.05) as compared to respective Control .

3)Parameter of sperm

The results in Figure (7,8,9,10,11) showed the effect of Etoposide , Quercetin and Hesperidin on sperm parameters in male rats during the study period , Where showed of results significant decrease (P<0.05) in the sperm count ,sperm of motility , the sperm of activity, the percentage of sperm viability and the percentage of normal sperm in male rats treated with Etoposide (T1) when compared with the control group and treatments groups (T2, T3 and T4) .

The results in (Figure 7) showed a significant decrease (P<0.05) in the sperm count of the groups (T3 and T2) compared with control group and T4, while no significant difference (T2 and T3) showed significant differences (P<0.05) , While the difference between control and T4 did not reach the moral level (P> 0.05).

The results (Figure 8) of the study showed a significant decrease (P <0.05) in the percentage of the Motility sperm of the T2 and T3 groups compared to the control group and the T4 group. The differences between T2 and T3 were not significant when comparing each other significantly (P> 0.05). The results did not show a significant difference (P>0.05) between control and (T4). In the results of the degree of sperm activity(Figure 9)   there was a significant decrease (P> 0.05 in treatments (T2, T3, T4) when compared with control as well as with each other .
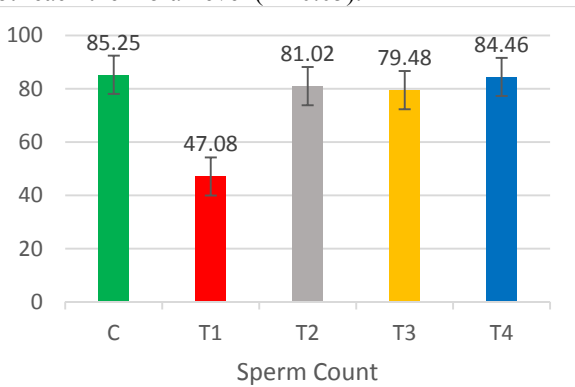

Figure 7: Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on sperm count .
 Values are expressed as Mean ± SE for five animals in the group.
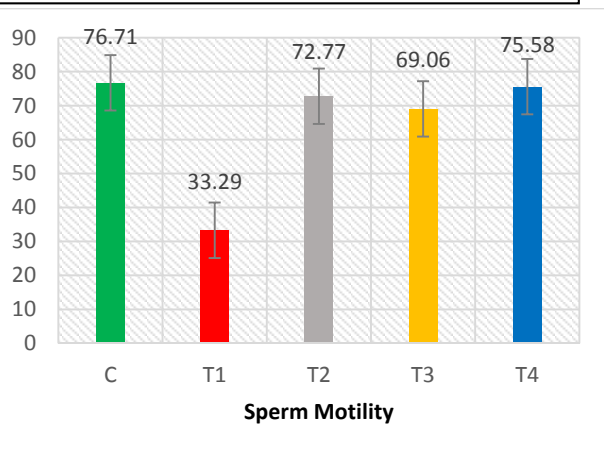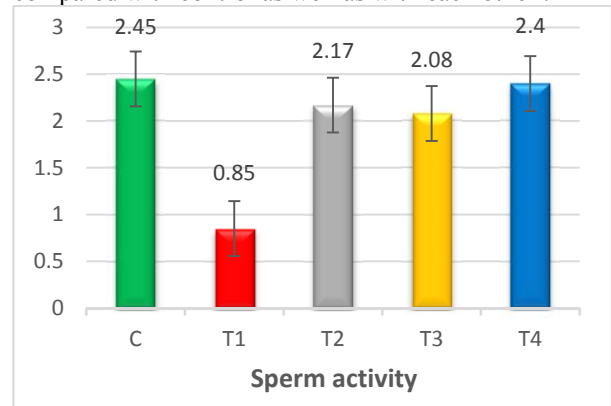- Different small letters are different significant (P<0.05) as compared to   respective Control .

Figure 9: Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on sperm activity .

 Values are expressed as Mean ± SE for five animals in the group.

- Different small letters are different significant (P<0.05) as compared to   respective Control .

Figure8   : Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on sperm motility
 Values are expressed as Mean ± SE for five animals in the group.
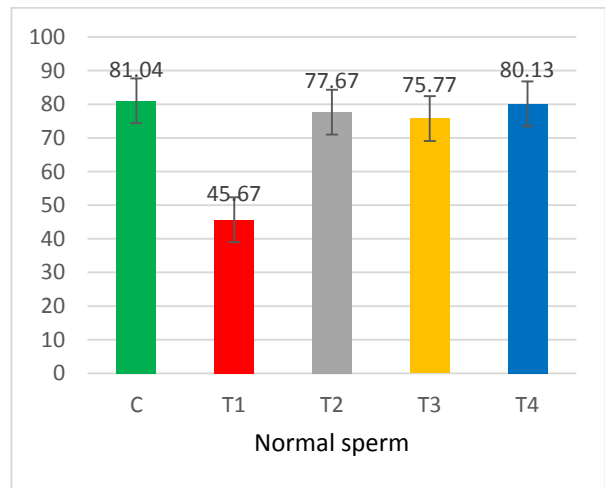- Different small letters are different significant (P<0.05) as compared to   respective Control .

Figure10 : Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on normal sperm.

 Values are expressed as Mean ± SE for five animals in the group.

- Different small letters are different significant (P<0.05) as compared to   respective Control .

The results (Figure 10) of the percentage of normal sperm showed a significant decrease (P <0.05) in the T3 group compared with the control groups and (T2 and T4), Also found Significant difference (P <0.05) in the T2 group compared with control group and (T4), while the difference between control group and T4 did not reach the moral level (P> 0.05).

The results of the percentage of live sperm (Figure 11) showed a significant decrease (P <0.05) in the T2 and T3 groups compared with the control and (T4). The results did not show a difference in the T2 and T3 levels (P> 0.05). When comparing control group and T4 ratios, no significant differences were found between them (P> 0.05).
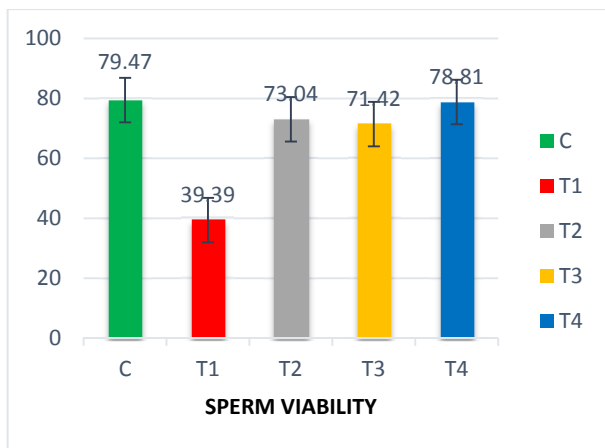

Figure11 : Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on sperm viability

Values are expressed as Mean ± SE for five animals in the group.

- Different small letters are different significant (P<0.05) as compared to respective Control .

Test Fertility :

Figure (12) shows that birth weights did not show a significant difference (P <0.05) in the animals treated with Etoposide (T1) compared to all groups . There was no significant difference (P <0.05) in the birth weight of male fertilized females experiment animals in treatments groups (T2, T3 and T4) when compared with control as well as with each other.

The results of (Figure 13) showed a significant decrease (P <0.05) in the number of births in the treatment group (T1) when compared to all groups, while a significant difference was observed (P> 0.05) when comparing the rates between treatments groups (T2 and T3) with control and (T4) , while there were no differences between them , (T4) didn't show a significant difference (P <0.05) when compared with control . The results in (Figure 14) showed a significant decrease (P <0.05) in the fertility ratio in the treatment group (T1) when compared to all groups, while no significant difference was observed (P> 0.05) (T2 and

T4) and control group, while the group (T3) showed a significant difference (P<0.05) when compared with control and with the rest of the groups.

Weight of birth

Figure12 : Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on birth weight

Values are expressed as Mean ± SE for five animals in the group.

- Different small letters are different significant (P<0.05) as compared to respective Control .

NUMBER OF BIRTH

Figure 13: Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on number of birth .

Values are expressed as Mean ± SE for five animals in the group.

- Different small letters are different significant (P<0.05) as compared to respective Control .
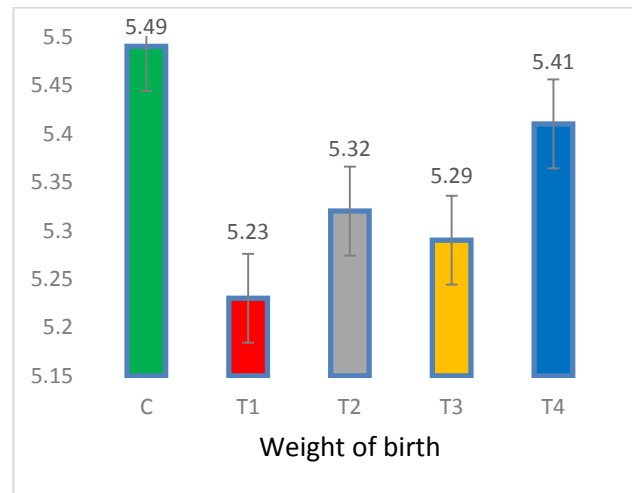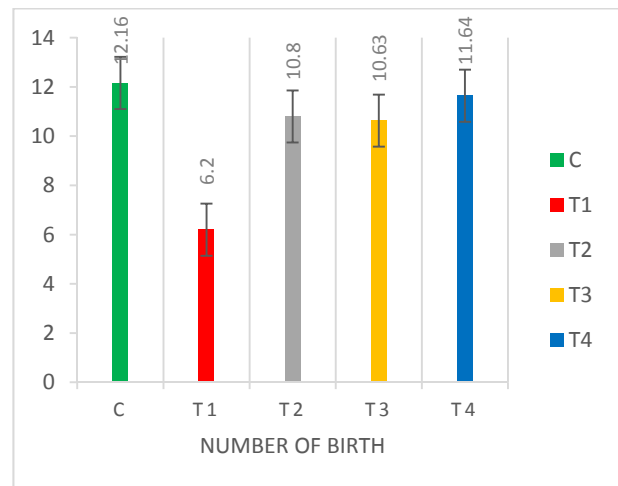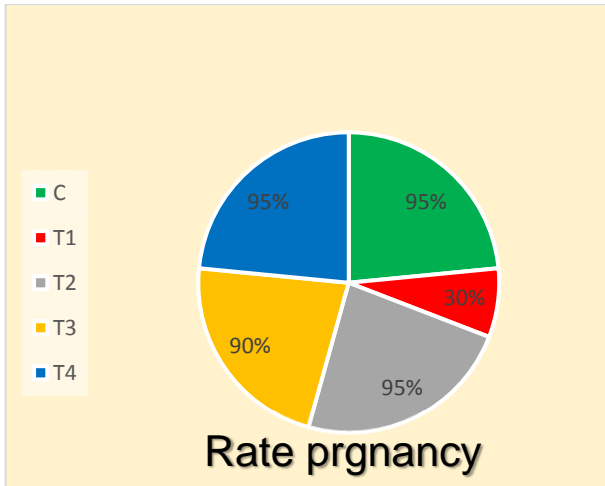
Figure 14: Effects of administration Etoposide , Quercetin and/or hesperidin in treated rats on rate pregnancy .

Values are expressed as Mean ± SE for five animals in the group.

- Different small letters are different significant (P<0.05) as compared to respective Control .

Figure 16 : real time PCR amplification plot for **CYP11A1** gene in testis show a difference in cycle threshold numbers (CT value) between treatment and control groups.

Red plot: Control group. , Blue plot: (T1) Etoposide group , Green plot: (T2) ETO + QE group , Yellow plot:
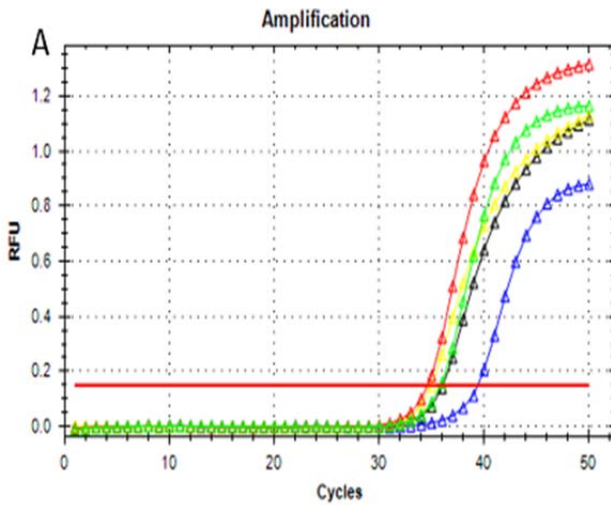
Figure 15 : real time PCR amplification plot for **LH receptor** gene in testis show a difference in cycle threshold numbers (CT value) between treatment and control groups.

Red plot: Control group. , Blue plot: (T1) Etoposide group , Green plot: (T2) ETO + QE group , Yellow plot:

Figure 17 : real time PCR amplification plot for **LH subunit** gene in testis show a difference in cycle threshold numbers (CT value) between treatment and control groups.

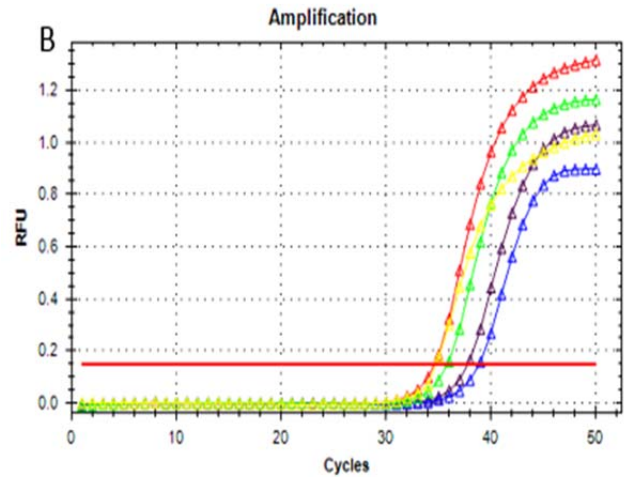Red plot: Control group. , Blue plot: (T1) Etoposide group , Green plot: (T2) ETO + QE group , Yellow plot:

Figure 18 : real time PCR amplification plot for **GAPDA** gene in testis show a difference in cycle threshold numbers (CT value) between treatment and control groups.

Red plot: Control group. , Blue plot: (T1) Etoposide group , Green plot: (T2) ETO + QE group , Yellow plot:
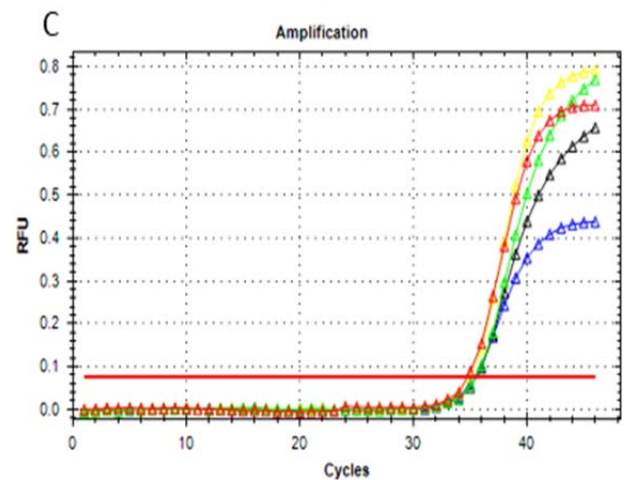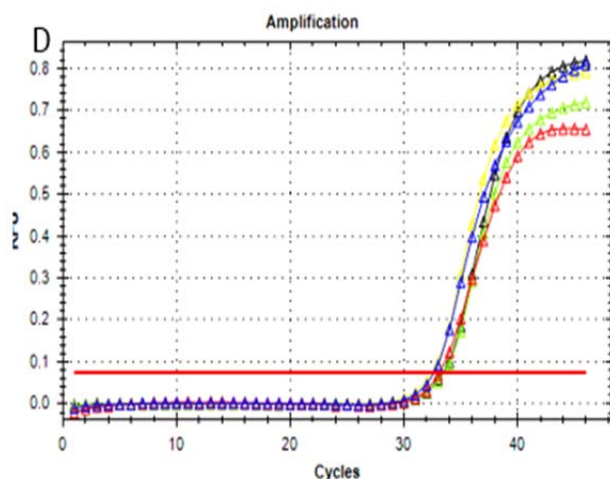
DISCUSSION :

The study was aimed to investigate the role of Quercetin and/or Hesperidin to improvement testis functions by using males rats as a model , Many studies have indicate to the negative effects of anti-cancer drugs properties such as free radical and oxidative stress and causes damage to the reproductive and other organs. In the present study, Showed a decrease of the expressions of steroidogenic enzymes in the testes and pituitary gland of rats exposed to Etoposide , Our current study was identical to previous studies in experimental animals treated with anti-cancer drugs (15 , 16 , 17) . Studies have reported that exposure to a wide variety of anticancer causes lipid peroxidation, which leads to oxidative stress , which effects drugs on pituitary-testis axis in cancer patients (17 , 18). These drugs included cyclophosphamide, doxorubicin, cisplatin, vincristine and many other drugs affected reproductive hormonal levels in male patients (19, 20 , 21). Previous reports have proposed that etoposide antimitotic activity is mediated by its interaction with topoisomerase II, an ATP-dependent nuclear enzyme that regulates DNA topology by transiently breaking and uniting double-stranded DNA (22, 23). Etoposide interacts with DNA-topoisomerase II, an enzyme present in cells undergoing rapid proliferation. In the testes, the etoposide targets are mainly intermediary and spermatogonia because they contain a lot of topoisomerase II (24)
However, current studies was evaluated the effects of Etoposide on testicular steroidogenesis at molecular level. In our previous study, administration of Etoposide resulted in a decrease in testosterone, FSH and luteinizing hormone levels suggesting the disruption of pituitary-testis hormonal

axis because of oxidative stress by Etoposide (25). In the present study, the reduced testosterone, LH and FSH levels at the end of treatment indicate oxidative stress -induced Leydig cell dysfunction, alterations in Leydig cells are also related to changes in the pituitary–testicular axis (26). Although many studies in cancer patients and experimental animals have shown altered pituitary–testicular hormonal axis (18, 27 , 28) , These hormonal changes formed a basis for the investigation of gene expressions of enzymes involved in cholesterol transport and steroidogenesis in same animals , on the other hand, inhibited the activity of Cyp11A1 enzyme, another rate-limiting enzyme in the testosterone synthesis pathway, is necessary for breaks cholesterol transport into a cell that is involved in steroidogenesis to produce pregnenolone in mitochondria Hence, even if cholesterol was abundantly available in mitochondria of the Leydig cells for side chain cleavage, the formation of pregnenolone would have decreased due to the unavailability of Cyp11A1 , and this may be one of the reasons for the low testosterone level seen in this group (29). It appears that the decreased luteinizing hormone level, possibly due to direct effects of Etoposide on pituitary gland (25). The decrease in the transcription of steroidogenic enzymes may be related to structural effects of Etoposide on both nuclei and cytoplasm of the Leydig cells. Nevertheless, the inhibited transcription of the genes have resulted in a decrease in serum testosterone level (25). Our study accompanied with (30) where considered that the cause of reduction in testosterone level of rats treated with anti-cancer treatments (etoposide, cisplatin, bleomycin) may be due to inhibition of Etoposide gene expression of steroidogenesis (Cyp11A1 , HSD3B1, HSD17B6) . This was confirmed by Sergejew and Hartmann (31) after 7 days of cisplatin in male rats, while Conley (32) reported that the reduction in the production of androgens was due to the inhibition of gene expression of Cyp17A1 and 17β-HSD also responsible for the steroid synthesis. Interestingly, our results are not in agreement with previous studies on effects of single exposure or 1 week-long exposure to high dose-levels (7–9 mg/kg) of cisplatin, which decreased testosterone levels, but not luteinizing hormone and follicle-stimulating hormone levels (33). But these authors reported that cisplatin could inhibit luteinizing hormone receptors (LHr) and CYP11A1 (CYP450scc) in rat testes (15, 33). It appears that the decreased luteinizing hormone level, possibly due to direct effects of Etoposide on pituitary gland (25) .
The results of this study revealed Etoposide administration significantly decreased sperm count, sperm motility, activity sperm ,Viability sperm and sperm morphology of the rats due to damage caused by free radicals from Etoposide – treated .The changes observed in the above agree with the previous reports, which demonstrated that anticancer drugs impairs testicular function (10, 34, 25) . lipid peroxide is an essential process in many pathological events and is caused by oxidative stress , lipid peroxide is considered as one of the basic mechanisms of cell damage caused by free radicals after being reacted with lipid causing oxidation , Which lead results in the release of

products such as malondialdehyde (MDA), Hydrogen Peroxide (H2O2) and Hydroxyl Radicals (35). These intermediates may be responsible for destroys the structure plasma membranes of sperm and makes it more fragile as a result of the breakdown of unsaturated fatty acids and are very sensitive to the ROS , and it is associated with sperm cluster and its non-motility, decrease in the percentage of live sperms and increased percentage of dead sperms , morphologically abnormal sperms and impairment of spermatogenesis , which leads to infertility (36 ; 37) . ROS that cause oxidation of group (-SH) in proteins and DNA that alter the sperm production and function and increase the Its susceptibility to attack by means of attachments (38), suggested study by Said (39) that abnormal sperm morphology combined with elevated ROS production may serve as a useful indicator of potential damage to sperm DNA . Oxidative stress has been implicated as a factor that contributes to various forms of cell death, including as a specific inducer of apoptosis by activating caspase-8, which reflects the effect on spermatogenesis (7). Many of Etoposide dose-limiting toxicities occur due to its generation of toxic oxygen species, resulting in oxidative stress, which negatively affects the cell division leading to death and then weakness fertility or infertility (40).. Shown studies (7, 41) findings the testicular alterations and germ cell damage caused by etoposide could be partially mediated by Sertoli cells which can provoke serious harm to spermatogenesis . The suggested mechanism is that etoposide up-regulates certain caspases, important regulators of the cell cycle, and this leads to induction of apoptosis in affected cells (42) .

In our study showed decrease in test fertility because decrease of testosterone , LH and FSH in the current study, which are the main responsible for the composition spermatogenesis and development of sperm (Bieber et al., 2006) , or may be due to the damage caused to DNA germ cells can be caused oxidative stress by the treatment as spermatogonia and up to the premeiotic differentiation but not during the post-meiotic steps , this may be explained with the high frequency of cells with the features of programmed death due to inhibition of Topomearse II , causing damage in the sperm cell DNA, which forces the cells to commit suicide in the process of death the programmed cell apoptosis led to decreased spermatogenesis , and possibly the damage to this DNA can be passed on to generations of treated animals, and can lead to death in births (10) . This is the conclusion of our current study, Bieber et al. (34) reported that the exposure of rats to platinum -cis for 9 weeks caused an increase in the number of deaths and congenital malformations of the fetus , The current study agreed with previous studies (7, 34,10) .

Treatment with quercetin and/or Hesperidin improved the current study parameters , may be due to of Flavonoid compound help to normalize the affected testicular functions and the hormonal axis (43 , 44) . Led treatment with quercetin, the results show there is a significant increase in gene expression for CYP11A1, LHr and LH subunit gene as well as Testosterone and LH and FSH

,These results are in agreement with earlier reports (43 ;45). flavonoids that reported the protective potentials in improving the testicular and pituitary functions and maintaining the cellular components of DNA, RNA, nucleic acids and lipid in the membranes through the reduction of lipid peroxidation and increased antioxidants in the tissues (46,47). Also (48) is mentioned in the ability of flavonoids to protect the biopolymers from the effect of free oxygen-free radicals that cause damage to DNA , Miyake et al., (49) mention that Hesperidin improved the levels of GSH, CAT in diabetic rats and inhibited oxidative stress free radicals, and reduced oxidase 8-hydroxy-2' -deoxyguanosine (8-OHdG) derived from deoxyguanosine , an indicator of DNA oxidation , Hesperidin and Quercetin have improver effect on plasma gonadotropin concentration (50, 51) , Also quercetin have effect on testis by other sex organs through stimulating the testis and epididymis or hypothalamic-pituitary-testis axis through stimulating testosterone hormone secretion (47). In this study the reproductive hormones have cooperative effect on sperms and testis cells. LH stimulate leydig's cells to synthesis of testosterone hormone, and then come the role of FSH which is stimulate sertoli cells to production of androgen binding protein(ABP) , which transport of testosterone to the target site in the spermatogonia and epididymis for development and maturation of sperms (52). Quercetin also stimulates the enzymes responsible for the carry of cholesterol to Leydig cells for the synthesis of testosterone (53). Quercetin also influence the enzymes that convert cholesterol into pregnonolone, the first step in the synthesis of steroids (52) . Khaki (54) noted that Quercetin caused an increase in hormones (Testosterone, LH, FSH) in diabetic rats with streptozotocin . Indicate study of (55) clearly demonstrated that hesperidin have attenuated the harmful effects on reproductive system toxicity of cisplatin-induced in male rats . Hozayen et al., (56), showed pretreatment hesperidin improved (testosterone, LH and FSH) level in serum in rats, improvement testicular toxicity caused by doxorubicin – induced .

In the present study, administration of QE (20 mg/kg bw) and/or HES (25 mg/kg bw) /rat for 2 months significantly increased sperm motility , count , activity , viability and normal morphology in both experimental groups as compared with the Etoposide-treated group could be due to the protective effect of flavonoids (QE , HES) administration , which have been reported to scavenge H2O2, hydroxyl radical , nitric oxide , superoxide anion . Thus, in inhibiting the cellular DNA damage (57, 58).

Several studies have reported the inhibitory effects of catechin, quercetin, and other flavonoids on in vitro lipid peroxidation. Beside, these productive effects are reflected by the decrease of malonaldehyde level and increase in total antioxidants capacity in according with these results, ElMazoudy et al., (59) Showed the positive effect of quercetin (10 and 20 mg/kg) on sperm characteristics was observed as indicated by the increment in SOD and CAT activities because is ability of quercetin, via two aromatic rings in its structure, can penetrate the phospholipid membranes and upregulation of

antioxidant (60). Besides, it decreased the total abnormal sperm number in experimental groups . Similarly, Taepongsorat *et al.,* (61) observed that Quercetin improved the sperm motility, concentration and viability in rats . This finding is consistent with previous reports which indicated that quercetin increased the antioxidant activities of SOD and CAT (62), In study Trivedi *et al.,* (63) showed Hesperidin protects testicular toxicity of doxorubicin in male rat, prevention of oxidative stress, DNA damage and the cellular toxicity and protection against doxorubicin-induced germ cell toxicity was further evident from the sperm count and sperm head morphological evaluation. was confirmed (63) role of nuclear factor-kappa B, p38 and caspase-3 on hesperetin-mediated protection against doxorubicin-induced testicular toxicity.

In addition, the effect of Quercetin as an antioxidant and its ability to improve the function of mitochondria and cell movement and activity in general is likely to be achieved by maintaining the balance of calcium inside and outside the cell (64) , as explained by Ikizler *et al.,* (65) reported that Quercetin stimulates the increase in ATP production, which is the main source of energy and the important mediator of many vital pathways in the cell, activating the sperm movement.   study of (55) was observed that hesperidin was orally administered (50 mg/kg) for 14 days have attenuated the harmful effects on sperm characteristics of   cisplatin-induced reproductive system toxicity . Reported (66) that Hesperidin has proven ameliorative effects in BaP-induced testicular toxicity by increase sperm count, motility, and daily sperm production and this protection resides, at least in part, on its antioxidant properties.

Showed our study improved in fertility parameters , This may be due to the role of flavonoids(Hesperidin , Quercetin) in providing protection against oxidative stress and damage caused by free radicals at the tissue level and various body organs including the male reproductive system, This is consistent with the results of some studies that demonstrated the importance and efficacy of flavonoids in reducing the toxicity of anticancer cancer-induced in fertility parameters (47, 55) .   It also works Quercetin to inhibit the enzyme Xanthine oxidase, which is the main enzyme in the process of lipid peroxidation and thus prevents this process and protects the cell membrane and mitochondria , This enzyme is one of the enzymes that mediate the process of generating free radicals(59) . The current results clearly indicate that Quercetin induced fertility as indicated by a marked increase in the testosterone and LH and FSH levels. Similarly, Khaki *et al.,* (54) found that Quercetin increased the serum testosterone levels and had beneficial effects on sperm parameters in streptozotocin-induced diabetic male rats. Also, Ma *et al.,* (47) reported that Quercetin can increase serum testosterone levels in male rats .

Combining antioxidants may increase their effectiveness , In (QE +HES) showed synergistic potential and significant reach all parameters in current study to control group , recently reported attempts to elucidate the mechanism of action of this Flavonoids, due to have the ability to form non-covalent bonds within the lipid layers close to the cell's plasma membrane, Which greatly enhances its antioxidant activity in this combination and regeneration of endogenous antioxidants (67). The flavonoids may interact with the polar head of phospholipids at water lipid interface, enhancing membrane rigidity and consequently protecting membranes from oxidative damage (68) , The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure. The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability (69) , Conjugation between the A (in Quercetin) and B (in Hesperidin) rings allows a resonance effect of the aromatic nucleus that provides stability to the flavonoid radical . Free radical scavenging by flavonoids is potentiated by the presence of both the elements besides other structural features (70). It is proposed that B ring OH groups form hydrogen bonds with the 3-OH, aligning the B ring with the heterocycle and A ring. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is enhanced by the presence of a $3',4'$-catechol, elucidating the potent antioxidant activity of flavan-3-ols and flavon-3-ols that possess the latter feature (71,72), Flavonoid protect against oxidative damage by various mechanisms include (1) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation; (2) scavenging ROS; and (3) upregulation or protection of antioxidant defenses (70) .

Some researchers have reported synergy between naturally occurring flavonoids in vitro   , vivo of animals experimental . Smet *et al.,* (73) found that dietary synthetic antioxidants combined with α-tocopherol were more effective than rosemary, green tea, grape seed, or tomato extracts alone, in sparing tocopherols oxidation and in preventing oxidation of fresh frozen chicken patties . In a study by Hozayen *et al.,* (74), found that pretreatment with rutin and/ or hesperidin (synergism of Flavonoids) may improvement of testicular dysfunction caused via doxorubicin – induced, by amelioration in the levels of (testosterone, LH and FSH) in male rats . Schlachterman, *et al.,* (75) was determined that combined dietary polyphenols (Quercetin, and Catechin) led to inhibit breast cancer progression growth in a nude mouse model in vivo , indicate Zhang *et al.,* (76) were Combination of curcumin and quercetin has the potential than that of individual treatment as anti-gastric cancer drug for further development, through inhibit the phosphorylations of ERK and AKT and induce apoptosis via mitochondrial pathway .

CONCLUSION

The findings in this study showed that Flavonoids enhances testicular oxidative status through administrated (QE) and (HES) severally or in combination (synergism) prevents by improved the harmful effects of Etoposide on reproductive parameters in male rats and toward the normal values .

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