

*Original Article*

# Evaluating the Influence of Rosemary Leaves Extract on Hormonal and Histopathological Alterations in Male Rabbits Exposed to Cypermethrin

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## Abstract

Rosemary Leaves (*Rosmarinus officinalis*) gained importance as natural antioxidants which strengthen the endogenous antioxidant defenses through die. The present experience was designed to assess the protective effect of ethanolic extract of rosemary leaves on the adrenal gland and testicular toxicity in male rabbits exposed to Cypermethrin. Forty healthy male rabbits were distributed into four groups of 10 animals each; the animals were administered cypermethrin 66.5 mg/kg alone or concurrent with Rosemary extract in both dosages (100 and 200 mg/kg) for 45 days, and the blood samples were taken from all animals for estimation hormones indices, the Anaesthetized animals were euthanized and adrenal gland and testes were separated for histopathological analysis. Results revealed that the exposure to Cypermethrin induced stress and infertility as evidenced by elevation in the level of cortisol concurrently with a lowering in ACTH level. Also, recording elevation in FSH and LH levels and a significant decline in estradiol level related to a reduction in testosterone levels observed noticeable compared to healthy control. While Concurrent exposure to Cypermethrin and Rosemary extract significantly improved hormone criteria compared to rabbits exposed to Cypermethrin alone. Histological lesions in this study include: the adrenal gland appeared thick fibrous capsule surrounding the adrenal tissue, destruction of adrenal cortex and vacuolation of three layers of the cortex, while in testes marked inhibition of spermatogenesis and degeneration of Sertoli cells with few numbers of Leydig cells were shown. These alterations were brought about by cypermethrin toxicity, while the treatment of Rosemary leaves extract with Cypermethrin alleviated the deleterious effect of Cypermethrin on the adrenal gland and testes and also restored spermatogenesis. The results showed that the extract of rosemary leaves possesses anti-infertility and strong antioxidant activities and can be used as a fertility-increasing drug to control sexual hormones also spermatogenesis, preventing toxicity and its pathophysiological consequences.

**Keywords:** Rosemary leaves, ACTH, Cortisol, testosterone, E2, Adrenal gland

## 1. Introduction

Synthetic pyrethroids (pesticides) are endocrine-disrupting chemicals (EDCs) that cause impairment of fertility in males. Also, it is accompanied by health risks to livestock and humans (1). They are derived from pyrethrins, which are found in the flowers of *Chrysanthemum cineraria folium*; regard to their chemical structure, the pyrethroids are comprised of (2

types); the first type of pyrethroids cause hyper-excitation as well as affect the sodium channels in the closed state and fine tremors such as permethrin, resmethrin, tetramethrin, bioemethrin, and allethrin, while the second type of pyrethroids induce high complex syndrome and affecting the sodium channels in the open state such as deltamethrin, Cypermethrin (CYP), cyfluthrin and cyphenothrin (2). Cypermethrin

(type II pyrethroids, alpha-cyano, molecular formula of  $C_{22}H_{19}Cl_2NO_3$ ) is commonly used as an ectoparasiticide in animals, in a public health setting, and in controlling pests (3). Some studies have observed the adverse effect of  $\alpha$ -CYP on the insect's nervous system; it causes neurotoxicity in mammals and long-lasting prolongation of sodium permeability during excitation. This neurotoxicity may cause severe repetitive nerve impulses in the sensory organs and damage the voltage-dependent sodium channel. This damage causes it to stay open much longer than normal; therefore, it is possible to generate excessive reactive oxygen species (ROS) following exposure to CYP (4, 5). The  $\alpha$ -CYP is metabolized in the liver by cytochrome P-450 enzymes that produce ROS formation, hence further production of ROS causing cytotoxicity and genotoxicity in organisms.

Furthermore, during  $\alpha$ -CYP metabolism, cyanohydrins are analyzed more than aldehydes and cyanide materials. These products can be stimulated to ROS formation (4). Exposure to Cypermethrin can be through ingestion or inhalation, and through dermal exposure, it is rapidly absorbed in the body, causing a state of physiological stress, damage to DNA and chromosomal aberrations, as well as disruption of spermatogenesis, neurotoxicity, and respiratory failure (5). Since common medical treatments have side effects, especially in association with their long-term use, and have high costs imposed on patients, a tendency towards alternative and traditional treatments is increasing (6).

Rosemary (*Rosmarinus officinalis L.*), which belongs to the Lamiaceae family, is an aromatic herb, increasingly used in food processing as a flavoring and spice agent; in recent years, its extracts have been widely used in the treatment of several diseases and cosmetics (7). Rosemary is a source of bioactive phytochemicals as it contains various phenolic compounds such as carnosol, carnosic acid, rosmanol, 7-methyl-epirosmanol, isorosmanol, rosmadial, and caffeic acid, principally the phenolic compounds. The flavonoids are responsible for Rosemary's antioxidant

and anti-inflammatory properties (8). Rosemary leaves its antioxidant activity by limiting the extent of lipid peroxidation and restoring the optimal balance by neutralizing the reactive species (ROS) by donating H (2 -OH groups) or by scavenging harmful free radicals and activation of physiological defense mechanisms (6). Lo, Liang (9) demonstrated that carnosol, a naturally occurring polyphenol found in rosemary leaves, showed potent antioxidative activity against *o*-diphenyl-B-picryldrazyl free radicals produced from the Fenton reaction. Rosemary leaves also contain other active constituents that have antioxidant properties, such as alkaloids, tannins, glyceric acid, glycolic acid, vitamin C, vitamin B, and choline (10). Leaves of Rosemary possess anti-carcinogenic and anti-mutagenic properties. The Rosemary leaves are also helpful in treating or preventing hepatotoxicity, spasmogenic disorders, atherosclerosis, peptic ulcer, bronchial asthma, and renal and heart diseases (11). The current study aimed to assess the protective effects of ethanolic extract of Rosemary leaves on the adrenal gland and testicular toxicity in male rabbits exposed to Cypermethrin.

## 2. Materials and Methods

### 2.1. Preparation of Rosemary Ethanolic Extract

Rosemary leaves were purchased from the local market in Thi-Qar, Iraq, and ground to a fine powder. Extraction of Rosemary was carried out according to the method of Abbasi Oshaghi, Khodadadi (12). 50 g of the delicate powdered herb were mixed with 200 ml of ethanol 70 % put in the flask round-bottom, left to boil slowly for 12 hrs using a reflex extractor. The mixture was filtered through Whatman filter paper (No. 31), and the alcohol was evaporated under vacuum using a rotary evaporator to yield the pure extract. The final product was left in the petri-dish under the shade to get the residues, and the dry extract was stored at 4 °C until used.

### 2.2. Experimental Design

Forty adult male rabbits with rang weights (1250-1300 g), animals were brought from honor in the local

market of the Thi-Qar and housed in the plastic cages of the animal's house that was maintained under a temperature- ( $25 \pm 2$  ° C) and 12 hrs (light/ dark) cycle with the controlled room ventilation. The animals had access to an ordinary diet and tap water *ad libitum*; rabbits were an adaptation for 2 weeks before experience. The rabbits used in this study were randomly distributed into 4 groups, each contained ten rabbits, and represented an experimental group as follows:

**Group I** Animals were orally administered corn oil and served as a control group for 6 weeks.

**Group II** (group CYP): Animals of this group were orally administrated CYP at a dose of (66.5 mg/kg b.w) dissolved in corn oil daily for 6 weeks.

**Group III** (Rosemary extract+ CYP): rabbits were orally given Rosemary extract at a dose of (100 mg/kg b.w) concurrently with oral administration of CYP at a dose of (66.5 mg/kg b.w) dissolved in corn oil daily for 6 weeks.

**Group IV** (Rosemary extract+ CYP): rabbits were orally given Rosemary extract at a dose of (200 mg/kg b.w) concurrently with oral administration of CYP at a dose of (66.5 mg/kg b.w) dissolved in corn oil daily for 6 weeks.

### 2.3. Blood Samples

24 hrs post the last dose of the treatment (45 days), fasting blood samples (about 5 ml) were collected via heart puncture of animals in each group. Samples were put in non-heparinized tubes and allowed to clot. Then all samples were separated by centrifugation at 300 rpm for 10 min. The serum was stored at  $-20$  °C for hormone assays. Anesthetized rabbits were euthanized, and isolated the adrenal gland and testis from treated rabbits and their control. The tissues were washed with normal saline, fixed using 10 % formalin, and prepared for histological examination.

### 2.4. Determination of Hormones

The level of follicle-stimulating hormone (FSH), luteinizing hormone (LH), total testosterone, and estradiol (E2) in serum samples were evaluated

according to the method previously described by Sharma, Huq (13). Cortisol hormone concentration was measured according to a method described by Hontela (14). While, Adrenocorticotrophic hormone (ACTH) concentration, ELISA kit was used (Guangzhou Wondfo Biotchco, LTD. China) according to the method previously described by Raff and Findling (15).

### 2.5. Histopathological Examination

Experimental rabbits and their controls were euthanized after 45 days of treatment. Their adrenal gland and testes were removed and fixed in 10 % neutral buffered formalin. The specimens of tissues (adrenal gland and testes) were put in ascending grades of ethanol, cleared in xylol, and then the tissues were embedded in commercial paraffin wax, and sections (5  $\mu$ m thick) were preparatory using microtome and stained by hematoxylin and eosin (H&E) for histopathological investigation.

### 2.6. Statistical Analysis

The data were represented as mean  $\pm$  SD of different groups, and the difference between mean values was evaluated by one-way analysis of variance (ANOVA); the statistical significance was analyzed at p ( $p < 0.05$ ) value.

## 3. Results and Discussion

Our results, illustrated in table 1, show a significant decrease in serum ACTH level while a significant increase in cortisol level in rabbits exposed to Cypermethrin compared with healthy control. This may be due to continued exposure to Cypermethrin for 45 days, causing a stress state. Furthermore, cortisol is a stress hormone that regulates its release via a negative feedback loop by altering ATCH release at the pituitary and hypothalamus; hence, under stress conditions, cortisol production increases associated with lower circulating ACTH levels significantly (16). These findings are the same as results obtained by Jin, Wang (17) demonstrated that exposure to cypermethrin stress causes prolonged cortisol elevation, finally creating negative feedback on the hypothalamic-pituitary-

interrenal (HPI) axis, resulting in suppression of ACTH release. The results also agree with the results of a study conducted by Ji, Song (18), who stated that pesticide exposure induces oxidative stress and endocrine disruption in male mice. Previous studies described by Hontela (14) and Marentette, Tong (19), have shown that the elevated serum cortisol levels indicate that the organochemical exposure induces a stress state hence cause to suppression on the HPI axis at the level of CRH, ACTH, well as downregulating receptors and causing atrophy of cells (pituitary corticotrophs), which is the common target of several environmental pollutants ranging from pharmaceuticals to pesticides, this will possibly can be reflected in the cortisol response. Also Kim, Kabir (20) found that the elevation in cortisol level indicates that the animal is under the stress of a condition (acute or sub-chronic), whereas a reduction in cortisol level indicates impairment of the HPI axis or interrenal exhaustion. According to the current study results, the administration of Rosemary extracts with cypermethrin cause to mitigates the adverse effect of Cypermethrin on the hypothalamic-pituitary-interrenal (HPI) axis, thereby improving the cortisol and ACTH release. Rosemary contains many phytochemical compounds, such as polyphenols, flavonoids, triterpenes, rosmarinic acid, ursolic acid, and carnosic acid. Therefore, it is a source of natural antioxidants (21). The recorded data were similar to Labban, Mustafa (22), which showed

that Rosemary acts as a cytoprotective agent against ROS. Rosemary extract can donate electrons to ROS and convert them to more stable molecules, preventing them from reaching biomolecules, increasing the normal cell viability and the antioxidant enzymes activity SOD and GSH, and significantly decreasing MDA content. Also, the findings are assisted by those found by Sakr and Lamfon (10), who observed that the anti-inflammatory and antioxidant properties of rosemary extracts are principally attributed to rosmarinic acid (RA) and carnosic acid (CA). The results recorded clearly in table 2 demonstrate that exposure to Cypermethrin for 45 days cause to disturbance in male fertility and reproduction, the male sexual activity regulation carried out by negative feedback loop including HPT axis (hypothalamus, anterior pituitary and testicles, it is evident that herein remarkable raise of serum FSH and LH levels in concomitantly with declined in level of testosterone observably, these changes of the hormones (FSH, LH and testosterone) in studied rabbits due to action of Cypermethrin on the testicular tissue and affecting the androgen synthesis by interstitial cell, then an increase in releasing of gonads hormones (FSH and LH) from the anterior pituitary due to feedback signal from significantly decreased in testosterone production thereby raising FSH and LH secretion as consequence of the adverse feedback action or due to the direct effect of Cypermethrin on the central nervous system (CNS) (1).

**Table 1.** Serum adrenocorticotrophic hormone (ACTH) and cortisol hormone in the control group and treated groups with Rosemary extract plus CYP in male rabbits

Parameters Groups	ACTH (pg/ml)	Cortisol ( $\mu$ g/dl)
Control (1mlcorn oil)	29.76 $\pm$ 1.89 a	17.32 $\pm$ 1.59 c
Cyp (66.5mg/kg)	22.91 $\pm$ 1.44 c	32.27 $\pm$ 3.35 a
RE (100mg/kg plus Cyp 66.5mg/kg)	23.65 $\pm$ 0.62 c	23.56 $\pm$ 4.14 b
RE (200mg/kg plus Cyp 66.5mg/kg)	26.9 $\pm$ 1.04 b	19.77 $\pm$ 0.85 c

Data are expressed as mean  $\pm$  SD (n=10). The different letters refer to significant differences ( $p \leq 0.05$ ) Cyp: Cypermethrin, RE: Rosemary ethanolic extract

**Table 2.** Serum FSH, LH, testosterone, and estradiol in the control group and treated groups with Rosemary extract plus CYP in male rabbits in male rabbits

Parameters Groups	FSH ng/ml	LH ng/ml	Testosterone ng/ml	Estradiol pg/ml
Control (1mlcorn oil)	10.50 ±1.19 c	0.90 ±0.02 c	7.35 ±0.48 a	1.98 ±0.36 a
Cyp (66.5mg/kg)	22.12 ±2.13 a	3.22 ±0.43 a	3.25 ±0.38 d	0.89 ±0.03 c
RE (100mg/kg plus Cyp 66.5mg/kg)	14.75 ±1.86 b	1.57 ±2.34 b	5.32 ±0.42 c	1.31 ±0.26 b
RE (200mg/kg plus Cyp 66.5mg/kg)	11.46 ±1.05 c	1.33 ±0.19 b	6.62 ±0.38 b	1.81 ±0.05 a

Data are expressed as mean ± SD (n=10). The different letters refer to significant differences ( $p \leq 0.05$ ) Cyp: Cypermethrin, RE: Rosemary ethanolic extract

Moreover, the elevation of urinary metabolite value of pyrethroid was correlated to the increase in FSH and LH concentration and lowering in inhibin B and androgen concentration (23). The results of this study were in agreement with results described by Solati, Hajikhani (24), who found that exposure to Cypermethrin at low doses (10, 15, 20 mg/kg i.p) induced a reduction in testosterone levels while increasing levels of FSH and LH in male mice. Also Ye, Li (25) observed in male rats that exposure to Fenvalerate (Fen, a type II pyrethroid) with a dose of 12 mg/kg caused decreased testosterone production while increased levels of FSH and LH as noticed in our study.

Another study showed that exposure to Cypermethrin at a dose of 60 mg/kg-day caused to decreased in testosterone levels, whereas with increased FSH level, the release of FSH from the pituitary significantly increased in response to lower production of inhibin B from Sertoli cells due to an adverse feedback action, or as a consequence to damage of the seminiferous tubules (26). Also Santos, Piccoli (27) demonstrated that short-term and long-term exposure to pesticide (lambda-cyhalothrin) accompanied increasing male LH values in the agricultural population in the South of Brazil. It is also clear from table 2 that exposure to Cypermethrin produced a reduction in estradiol levels related to a decline in circulating testosterone levels. This may be attributed to that CYP acts directly on the testicular and affects the androgen synthesis in the interstitial tissue. Abnormal interstitial cell lead to decreased

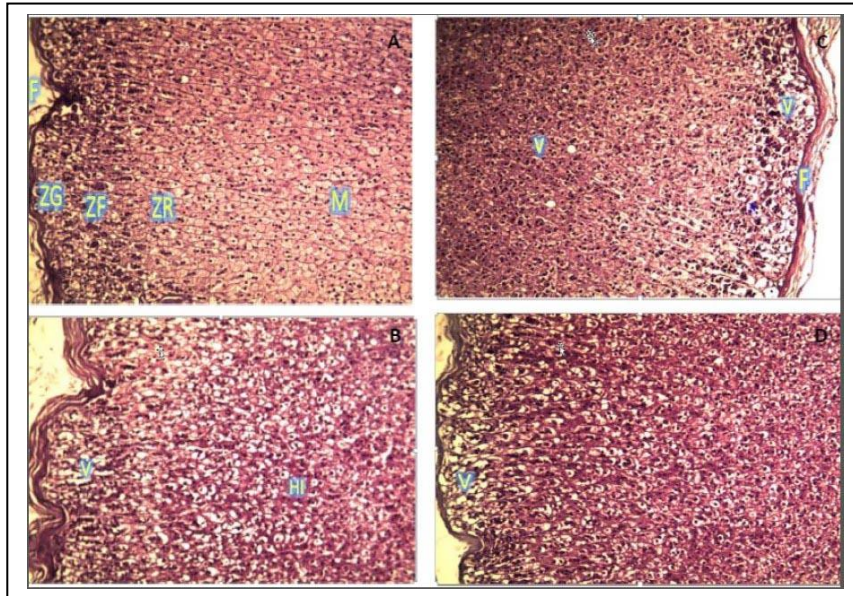
steroidogenic activity of the testicular (1). The results of the current study were consistent with Sharma, Khan (4), who observed that some pyrethroids act on the enzymes responsible for testicular testosterone syntheses like 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) and glucose-6-phosphate dehydrogenase causing inhibition of the enzymes thereby will interfere testicular testosterone synthesis. Also, Brander, Gabler (26) found that pyrethroids may exert an antiandrogenic effect via various pathways. Androgen receptors (AR) mediated signaling is a significant pathway. The pyrethroid binds to hormone receptors causing, blocking these receptors and then suppressive their action, as well as interfering with the synthesis, secretion, and action of androgen hormone in the testes, hence the low androgen level contributes to suppressive male fertility because of the vital role in the male sexual activity it is also seen in the table 2. The results (Table 2) exhibited that oral administration of rosemary extract plus Cypermethrin caused ameliorating testosterone production concurrently with the declined release of FSH and LH hormones. This beneficial effect is related to antioxidants' activity which prevents oxidative stress induced by Cypermethrin on the interstitial cells and germinal epithelium of seminiferous tubules, thereby increasing sexual functioning (6). The current results agree with results described by Modaresi and Emadi (28) demonstrated that rosemary extracts have antioxidant activity superior to alpha-tocopherol since alpha-tocopherol is a potent antioxidant that can prohibit oxidative stress in

the testis of the mice. Also, Sakr and Lamfon (10) reported that rosemary extract contains a high amount of sulfuric compounds, which have antioxidant and scavenger to free radicals properties, as well as its ability to remove the active metabolites of pesticides and cyclophosphamide drugs. In the same table, the results appear that the administration of rosemary extract caused to increase in estradiol levels that correlated to testosterone production. This may be attributed to rosemary extract having compounds that possess pro-fertility properties, which might be a product characterized by potent antioxidant and androgenic activity. The results agree with those obtained by Hamza, Al-Sharafi (29).

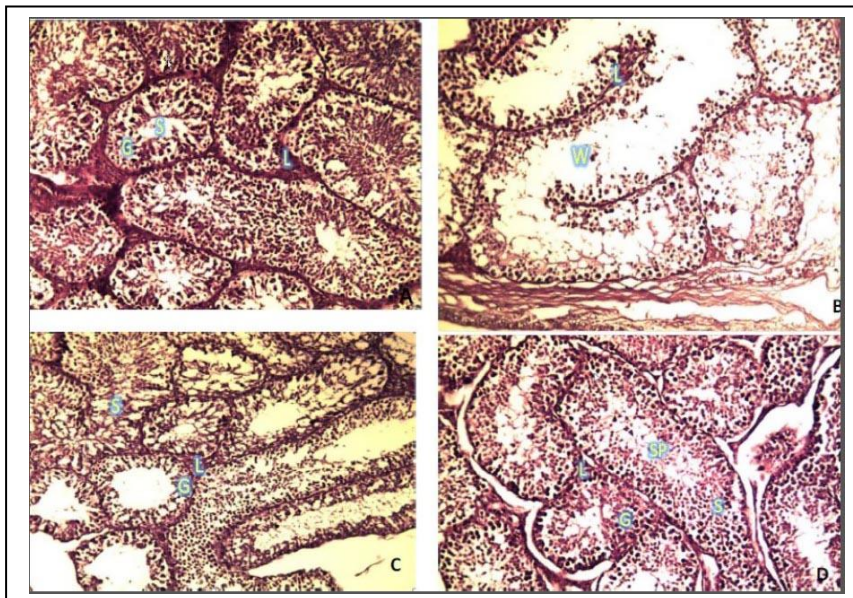
As regards the histological examination of the studied organs (adrenal gland and testes), the results went in the same line as hormonal changes. The administration of CYP for 45 days caused a significant histological injury to the adrenal gland, especially to the adrenal cortex and testicular tissue. The lesions are illustrated in figures (Figure 1B and Figure 2B) compared to standard histological features illustrated in figures (Figure 1A and Figure 2A). CYP induces oxidative stress and tissue damage; one possible mechanism may be the inhibition of mitochondrial ATP production through the uncoupling of oxidative phosphorylation leading to overproduction of free radical (ROS) and disturbed antioxidant capacity, and the toxicity of various pesticides as consequences to the ROS generation (17). In adrenal tissue, the lesions agree with previous studies. Ghassabian and Trasande (30) reported that exposure to pesticide (CPF) causes damage in the form of vacuolation of zona granulosa cells of the adrenal gland in rats. Lesions are also similar to those described by Settar, Oularbi (31), who found that the vacuolization, congestion, and proliferation in adrenal tissues of rabbits exposed to ampligoa synthetic insecticide.

Also, Abass, Elkhateeb (32) demonstrated that exposure to atrazine causes oxidative damage in the adrenal cortex and impairs its function in rats while in testicular. Similar lesions were observed in male rats exposed to CPF; CPF may exert oxidative stress on the testicular, increasing degenerative changes in the seminiferous tubules and interstitial cells and decreasing the interstitial cell number (33). A previous study conducted by Ngoula, Watcho (34) demonstrated that dimethoate causes testicular damage characterized by moderate to severe seminiferous tubule degeneration, sloughing, atrophy, and degeneration of germ cells, decreasing spermatogenesis. The antioxidant properties of Rosemary leave support its use in minimizing insecticide toxicity by modulating the redox reactions in adrenal and testicular tissues by decreasing the production of free radicals, which means increased antioxidant activity and improved antitoxic capability of tissues, the ameliorative changes related to adrenal and testicular tissues may be due to polyphenolic compounds, which may scavenge and inhibit ROS generation (35), illustrated in figures 1C and 1D and 2C and 2D. These findings are in the same line with findings obtained by Mwaheb, Sayed (6), reported that Plants rich in phenolic compounds may be used as dietary antioxidants to prevent oxidative stress induced-damage (36). Furthermore, the Anti-fertility effect of rosemary leaves, shown in testicular tissue illustrated in figures 2C and 2D, is supported by a previous study that reported that rosemary extract improves testosterone production and spermatogenesis (28).

The recorded data of the current study referred that the extract of rosemary leaves possesses anti-infertility and solid antioxidant activities and can be used as fertility increasing drug to control sexual hormones also spermatogenesis, preventing toxicity and its pathophysiological consequences.



**Figure 1.** adrenal gland section of male rabbits (control group 1A), There is a normal adrenal gland without any lesion, normal fibrous capsule (F)with normal (zona glomerulosa (ZG), zone fasciculate (ZF), the zona reticularis (ZR), medulla (M) (1B) The adrenal gland of male rabbits treated for 45 days, There is a thick fibrous capsule (F)surrounded the adrenal tissue and destruction with adrenal cortex and vacuolation of three layers cortex (ZG, ZF and), and hyperplasia (HI)of cells in the zona vascularis. (1C and 1D) adrenal gland section of male rabbits treated with rosemary extra (100 &200mg/kg BW) and Cypermethrin. There are normal adrenal layers with a normal thin fibrous capsule (F). The zona gromerulosa showed normal cells, and the thickness zona vscuaris showed mild vacuolation (V)and mild necrosis (N)10x H&M



**Figure 2.**Testis section of male rabbits (control group 2A), The seminiferous tubules showed normal, high cellularity and filled with sperms .normal spermatogonia (G)and high numbers of spermatocytes and normal numbers of laydig cells in the interstitium (L). (2B) male rabbits treated with Cypermethrin for 45 days, Marked inhibition of spermatogenesis in which there is severe vacuolation of spermatogonia and degeneration of Sertoli cells with few numbers of spermatocytes with a wide lumen of seminiferous tubules (W). Also, there are few numbers of Leydig cells (L)in the interstitial tissue. (2C and 2D)male rabbits treated with rosemary extra (100 &200mg/kg BW) and Cypermethrin for 45days. The seminiferous tubules showed compact, high cellularity and were filled with sperms .proliferation of spermatogonia (G)and high numbers of spermatocytes (SP), hyperplasia, and large numbers of laydig cells (L) in the interstitium .10 X H&E

### Authors' Contribution

Study concept and design: S. A. H.

Acquisition of data: S. A. H.

Analysis and interpretation of data: S. A. H.

Drafting of the manuscript: A. A. A.

Critical revision of the manuscript for important intellectual content: A. A. A.

Statistical analysis: A. A. A.

Administrative, technical, and material support: A. A. A.

### Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article according to the ethics committee of the University of Basrah, Basrah, Iraq.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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