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Adverse effects of cadmium on the thyroid, kidneys, and testes in Wistar albino rats and the possible modulatory role of *Zizyphus spina-christi* (Sidr) fruit extract (Histological and biochemical studies)

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ABSTRACT

Background: Cadmium (Cd) is an industrial and environmental pollutant that exerts adverse effects on different organs in humans and animals. Endocrine organs, such as the thyroid, kidneys, testis, and placenta, are sensitive to the toxic effects of Cd. *Ziziphus spina-christi* fruit has been claimed to exhibit potent anti-oxidative and free radical scavenging abilities. **Aim**: This work is mainly designed to evaluate the potential modulatory role of *Z. spina-christi* fruit extract against thyroid, kidney, and testicular impairments associated with cadmium chloride (Cd Cl2) exposure in rats. **Material & Methods**: Twenty-four male rats were divided into four groups (n = 6): group I served as control, group II was supplemented daily with *Ziziphus* fruit extract (200 mg/kg b.wt), group III was treated with CdCl2 (5 mg/kg b.wt) each other day, and group IV was administered with CdCl2 simultaneously with *Ziziphus* fruit extract. The experiment was conducted for 45 days. At the end of the experiment, the rats of all groups were weighed and sacrificed, and the thyroid, kidneys, and testes were immediately excised, and processed for histological and biochemical evaluation of antioxidants and apoptosis. Also, blood samples were collected and analyzed to estimate the appropriate biochemical parameters for each organ. **Results:** In CdCl2 treated rats, the levels of serum hormones (TSH, T3, T4, FSH, LH, and testosterone) were significantly decreased if compared with control. Additionally, the levels of calcium, phosphorus ions, and albumin were also decreased while the level of total cholesterol was significantly elevated compared with the control. Moreover, the levels SOD and GSH were significantly lowered while the level of MDA was elevated in the tissues of all target organs (thyroid, kidney, and testes). Also, the sections from the thyroid, kidney, and testes displayed pronounced deleterious histological changes. Furthermore, the mean % values of positively expressed cells for caspase-3 and annexin-v markers were significantly elevated in the thyroid and testicular tissues of CdCl2 received rats compared to control. Co-supplementation of *Z. spina-christi* fruit extract to CdCl2 exposed rats successfully restored the altered biochemical and histological and apoptotic changes induced by cadmium**. Conclusion:** It is concluded that *Z. spina-christi* fruit extract has a powerful protective role against Cd-induced deleterious biochemical and histological changes in the thyroid, kidneys, and testes of male rats.

Keywords: CdCl2, endocrine organs, antioxidants, hormones, histopathology, sidr extract.

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INTRODUCTION

Heavy metals produce a state of toxicity not only at high concentrations but also the low doses that accumulated in body tissues on long exposure can reach the toxic state **(Pouls, 2005)**. Cadmium (Cd) is a unique toxic heavy metal because of its low dosage toxicity, long biological half-life and low rate of excretion from the body, and its ability to be stored in tissues **(Barbier *et al*,. 2005).** Cd was classified as one of the most toxic environmental and industrial pollutants **(Manca *et al*., 1991)**. Cd can accumulate in the body over many years because the body hasn't a homeostatic mechanism to keep cadmium at a constant level **(Nasri, 2006).**

Cd has toxic effects on different mammalian organs, such as the brain, kidneys, liver, testis, and placenta **(Elkhadragy et al., 2018; Kim et al., 2018).** Also, Cd has an adverse effect on the thyroid gland **(Pilet *et al.*, 2002 and Pilet *et al.*, 2004).** In humans, Cd has deleterious effects on male and female fertility, breast, development, and cardiovascular system **(Maffinni *et al.* 2006)**. Cd exposure increased oxidative stress, endocrine disruption, and increased apoptosis in rabbits, dogs, and humans **(Takiguchi & Yoshihara, 2006; Siu *et al*., 2009).** Furthermore, Cd has been found to produce a wide range of biochemical and physiological dysfunctions in humans and laboratory animals **(Santos *et al*., 2004; Li *et al*., 2010).** Moreover, Cd can induce intracellular reactive oxygen species (ROS) production and lipid peroxidation, which may lead to tissue damage (**Gupta *et al*., 2004 and Kara *et al*., 2005).** Additionally, Cd treatment decreased antioxidant enzyme activities whereas it increased lipid peroxidation level and histopathological changes in the testes, liver, and kidneys of mice and cocks **(Manna *et al*., 2008; Li *et al*., 2010).**

Plant materials are significantly contributed to the improvement of human health in terms of cure and prevention of diseases **(Okoko and Oruambo, 2008).** There is much evidence that natural products and their derivatives have efficient anti-oxidative characteristics, consequently linked to anti-cancer, hypolipidemic, anti-aging, and anti-inflammatory activities **(Aruoma, 2003; Cho *et al*., 2006).**

Zizyphus spina-christi belongs to the family Rhamnaceae (**Abalaka *et al*., 2010)**, and widely grows throughout Upper Egypt and Sinai. Also, it is widespread in the Mediterranean region, Africa, Australia, and tropical **(Yossef *et al*., 2011).** *Zizyphus spina-christi* has a common name ‘‘Nabka or Sidr”, Arabs used it to maintain a healthy lifestyle **(Adzu *et al*., 2001)**. The genus *Zizyphus* (Rhamnaceae) is characterized by the abundance of phenolic compounds, especially flavonoids, anthraquinones, and tannins **(Shahat et al., 2001 and Tripathi et al., 2001).** These compounds have been described as strong antioxidant molecules (**Moreira et al., 2018).** The flesh of *Z. spina-christi* fruits is rich in carbohydrates, protein, fat, calcium, thiamin, riboflavin, niacin, and ascorbic acid **(Berry-Koch *et al*. 1990).** Other reports elucidated several active constituents of *Ziziphus spina-christi* like triterpenoid sapogenins, geranyl acetate, sterols, saponins, methyl hexadecanoate, peptide, cyclopeptide alkaloids, methyl octadecanoate, tannines, and flavonoids (such as rutin and quercetin derivatives) (**Jafarian et al., 2014; Kadioglu et al., 2016; Almeer et al., 2018).**

Zizyphus species are commonly used in medicine for the treatment of various diseases such as digestive disorders, weakness, liver complaints, obesity, renal disorders, skin infections, lost appetite, fever, pharyngitis, bronchitis, anemia, diarrhea, and insomnia **(Han and Park, 1986).** The genus *Zizyphus* is known for its medicinal properties as hypoglycemic, hypotensive anti-inflammatory, antimicrobial, antioxidant, anti-tumor, and liver-protective agent and as an immune system stimulant **(Said *et al*., 2006).**

The methanolic extract of *Z. spina-christi* is effective in reducing both hyperlipidemia and oxidative stress accompanying diabetes **(Hussein et *al*., 2006),** enhancement of endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px)) and glutathione reductase (GSH) in hepatic tissue as well as inhibition of malondialdehyde (MDA) **(Xiangchun *et al.*, 2009).**

Z. spina-christi extract has also been reported to possess a protective effect against aflatoxicosis and anti-conceptive properties in rats and have a calming effect on the central nervous system **(Abdel-Wahhab *et al*., 2007)**. Additional pharmaceutical applications of *Z. spina-christi* have revealed antifungal, antibacterial, antinociceptive, antioxidant, anti-diabetic, anti-plasmodial, anti-schistosomiasis, analgesic, and anticonvulsant activities among others **(El-Kamali and Mahjoub, 2009; Waggas and Al-Hasani 2010; Abalaka *et al*., 2011).**

Accordingly, the present work with mainly designed to evaluate the ameliorative role of *Z. Spina Christi* fruits against Cd Cl_2-induced toxicity in the thyroid, kidneys, and testes of male albino rats.

MATERIAL AND METHODS

1. **Chemical**: Cadmium chloride ($CdCl_2$) was purchased from Sigma Company in Cairo, Egypt.
2. **Preparation of *Ziziphus spina-christi* fruit** extract: *Ziziphus spina-christi* fruits were washed with distilled water, sun-dried for 3 days, and then crushed using a mixer. A 500 g of the powder was soaked in five liters of distilled water for 3days under constant shaking with intervals of 30 min. The mixture was filtered using 250 mm filter paper, and then the filtrate was freeze-dried at (-52°C). The obtained percentage of yield from the powder was 40% (200 g semisolid product). The obtained semisolid product was dissolved in the appropriate amount of distilled water.

3. Experimental design

In the present work, twenty-four males of Wistar albino rats weighing 180-200 grams were obtained from the Hellwan Breeding farm, Ministry of Health, Cairo, Egypt. For acclimatization, the animals were kept in plastic cages in a well-aerated room with the standard condition of illumination at a 12-hour light-dark cycle of temperature 25 ± 1°C and 50% relative humidity. They were provided with free excess tap water and a balanced diet *ad libitum*. After acclimatization for two weeks, the rats were randomly divided into four groups as follows, six male rats for each group (n=6).

Group 1 (control group): The rats were fed on a normal diet without any treatment.

Group 2 (***Ziziphus spina-christi*):** They were orally supplemented with a daily oral dose of 200 mg/kg b.wt of *ziziphys* fruit extract.

Group 3 (Cd Cl_2): They were treated orally with Cd Cl_2 (5 mg/kg B.wt) each other day (**Nna et al., 2017**).

Group 4 ($CdCl_2$ &*Ziziphus* fruit extract): The rats were treated orally with Cd Cl_2 simultaneously with *Ziziphus* fruit extract by the same previous doses. The experiment was conducted for 45 days.

4. Sample collection and tissue preparation:

At the end of the experiment, the fasted rats were weighed and sacrificed under diethyl ether anesthesia. Blood samples were collected; serum was separated by centrifugation at 860 Xg and kept frozen at -20^{o}C for further biochemical analysis. Rats were then dissected, and the thyroid, kidneys, and testes were removed. The right testis, kidney, and right half of the thyroid from each rat were processed for histological examination, while the left testis, kidney, and left half of the thyroid were preserved frozen for estimation of antioxidants and flow cytometric study positively expressed Annexin-v &cspase-3.

5. Investigated parameters

5.1 Serum analysis

i. Estimation of TSH, T3, and T4

Commercially available RIA kits were used to assay serum content of thyroid-stimulating hormone (TSH; rat-specific, Cat. No. RPA5541, Amersham Pharmacia, Piscataway, NJ), thyroxine, and triiodothyronine (T4 and T3, Respectively; Cat. No. 06B-254029, -256447, and -237124, respectively; ICN Biomedical, Aurora, OH).

ii. Estimation of FSH, LH, and testosterone:

The level of follicle-stimulating hormone (FSH) in serum was determined using rat follicle-stimulating hormone (FSH) ELISA KIT purchased from MyBiosoruce Company (Cat.NO. MBS281287). Serum luteinizing hormone (LH) level was measured using rat LH ELISA KIT purchased by My Biosoruce Company (Cat.NO.MBS2509833). ELISA KIT from MyBiosoruce Company was used to determine the level of testosterone in serum (Cat.NO.MBS026898).

iii. Estimation of calcium (Ca^{++}) and phosphorus ($PO4^{-}$)

Serum calcium was measured by spectrophotometric method (**Barnett** et al., **1973**) and serum phosphorus was determined according to **Goodmin (1970)**

iv. Estimation of Albumin and total cholesterol.

Serum albumin was determined according to **Doumans et al (1971**). Blood cholesterol was determined by the method of **Meiattini (1978**)

5.2 Measurement of tissue antioxidants (SOD &GSH) and MDA in thyroid, kidneys, and testes.

The activity of testicular, kidney and thyroid catalase (CAT) was estimated by using the rat CAT ELISA Kit **(Cat No. MBS2600683**) purchased from MyBiosoruce Company. Testicular, renal, and thyroid Glutathione reductase (GSH) activity was estimated by using a rat GSH ELISA Kit **(Cat No. MBS2600683**) purchased from MyBiosoruce Company. The level of Malondialdehyde (MDA) was measured by using a rat MDA ELISA Kit (Cat.NO.MBS738685) purchased by MyBiosoruce Company.

5.3 Histological examination of thyroid, kidney, and testis

The right testes, kidneys, and right lobe of the thyroid were removed from the rats of all experimental groups and fixed in 10% neutral buffered formalin, then dehydrated in an ascending series of ethyl alcohol. The processed tissues were embedded in paraffin wax. The prepared tissue blocks were sectioned on glass slides (5-6 μm thick) using a microtome. The paraffin sections were further processed and stained with hematoxylin and eosin **(Bancroft and Gamble, 2008**).

5.4 Flow cytometric study for Caspase-3 and Annexin -V

i. Flow cytometric detection of activated caspase-3 by a direct staining method

Flow cytometric detection of caspase-3 was done to check the number of apoptotic cells in the testicular and thyroid tissues for groups1, and 3&4. This technique is applicable where the fluorochrome is directly linked to the primary antibody (PE and FITC conjugate). The cells were prepared appropriately. The cell suspension was adjusted to a concentration of 1 × 10^6 cell/ml with PBS/BSA buffer (phosphate-buffered saline and 1% BSA). An aliquot of 100μ L of cell suspension was put into test tubes as required. The antibody (FITC rabbit anti-active caspase-3, solid as, material No.559341, catalog No. 554714, from BD Pharmingen) was added at the recommended dilution (10μ L for each sample), mixed well, and incubated at room

for 30 min. After that, the cells were washed with2 ml of PBS/BSA then centrifuged at 1500 rpm for 5 min, and discard the resulting supernatant. The cells were re-suspended in 0.2 ml of PBS/BSA or with 0.2 ml of 0.5% Paraformaldehyde in PBS/BSA if required. The data were acquired by flow cytometry. This analysis was performed in the Mansoura University Hospital using FACS (flow activated cell sorter) Calibur Flow Cytometer (Becton Dickin-son, Sunnyvale, CA, USA) equipped with a compact air-cooled low power 15 mW Argon ion laser beam (488 nm).

ii. Flow cytometry for annexin -v

Flow cytometric analysis was performed on FAC Scan (Becton Dickinson) using standard settings: fluorescence 1(FL1), 4 decades (logarithmic), detector 648 V, logs amplifier, compensation 1.1%; fluorescence 2, 4 decades (logarithmic), detector 496 V, log amplifier, compensation 22.8%. Data analysis was performed using lysis of software (Becton Dickinson). Biopsies from the testes and thyroid for goroups1, 3&4 of the studied groups were taken, and the cell suspension was prepared with Tris-EDTA buffer (pH7.4) (Sigma-Aldrich Co.). The cell suspension was fixed in ice-cold 96-100% ethanol (Sigma) at 4ºC overnight, centrifuged at 1,500 rpm for 10 min, and then re-suspended in PBS containing 50 µg/ml propidium iodide (PI) (Sigma-Aldrich Co.). For each sample, the analysis was based on the measurement of 10000 cells. Single-cell suspensions were prepared from the previous organs for at least six male rats and 1.5-3 ×106 cells were stained for expression of the designated lineage markers.

RESULTS

1. Body weight changes

In the *Ziziphus* feeding group, the mean body weight showed no significant change in comparison with control however, the mean body weight of CdCl2-induced rats was significantly lowered (P<0.001) if compared with control. Furthermore, in the cadmium-induced group simultaneously supplemented with *Ziziphus* extract, the mean body weight was significantly increased and showed no significant change with control. (Figure1)

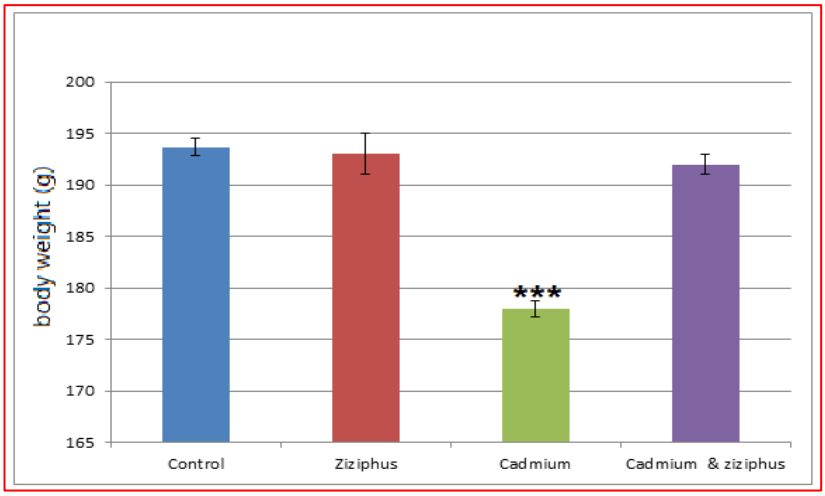

Fig.1. Shows the mean body weights (g) among the different studied groups. Note a highly significant decrease (***) in the mean body weight of $CdCl_2$ supplemented rats if compared with control.

Data are expressed as means ± Standard error, (n=6 per group). Statistical analysis: one-way ANOVA followed by post hoc test. Means in the same row with different superscript letters are significantly different (P<0.05).When P<0.05, *Significant at P-value ≤ 0.05, ** Significant at P-value ≤ 0.01and *** Significant at P-value ≤ 0.001.

2. The changes in hormones

In control and *Ziziphus* extract supplemented groups, the levels of serum TSH, T3 and T4 appeared in the normal standard range. In cadmium treated group the serum levels of TSH, T3, and T4 were significantly decreased (P<0.001) if compared with the control. On the other hand, the levels of serum TSH and T4 in the cadmium group co-supplemented with *Ziziphus* extract were restored to the normal value as control while the level of T3 still showed a low significant decrease (P<0.05) with control(figure2A). Further hormonal analysis revealed that the levels of serum FSH, LH, and testosterone of the cadmium-induced group were significantly decreased (P<0.001) if compared with the control. Supplementation of *Ziziphus* extract to cadmium-treated rats successfully restored the levels of FSH and testosterone to the normal value as control however the level of LH still with a low significant decrease (P<0.05) if compared to control (figure2B).

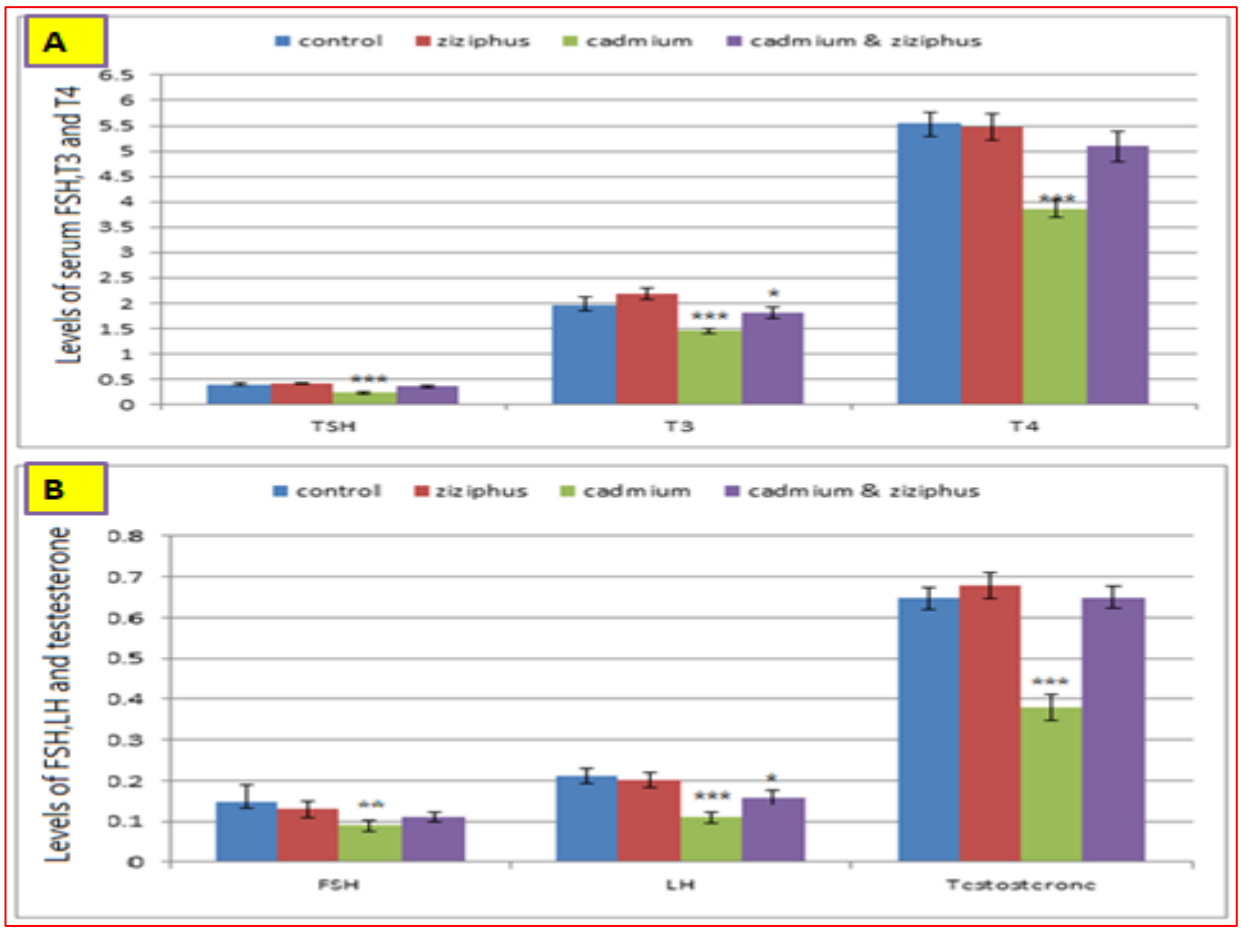

Fig.2: **A**: Showing the levels of serum TSH, T3, and T4 (pg/mL), **B:** showing the levels of serum FSH, LH (IU/L), and testosterone (ng/mL). Note: a highly significant decrease (***) of all hormones in the cadmium-group except FSH appears with a moderate significant decrease (**) if compared with control. In the cadmium and *Ziziphus* group, the levels of serum hormones restored near to the normal levels as in control except for T3 and LH still lower than control.

******highly significant, **moderately significant, * low significant***

3. *Changes* in serum albumin and cholesterol

In cadmium-induced rats, the level of albumin appeared significantly lower (P<0.001) however the level of cholesterol appeared significantly higher (P<0.001) if compared with control. On the other hand, the levels of albumin and cholesterol were significantly ameliorated if compared with the cadmium group but still showed low significant change (P<0.05) with control (figure3A&B).

4. Changes in serum Phosphorus and calcium

In *Ziziphus* extract supplemented rats, the levels of serum Ca^{+2} appeared significantly higher (P<0.001) than in control however a highly significant decrease of serum Ca^{+2} (P<0.001) was recorded in cadmium-induced rats. In the cadmium and *Ziziphus* group, the level of serum Ca^{+2} appeared significantly higher than control (figure 3C). On the other hand, a highly significant decrease (P<0.001) of phosphorus ion was recorded in cadmium treated group however a highly significant increase was noticed after supplementation of *Ziziphus* extract but still significantly lower than control (Figure 3D).

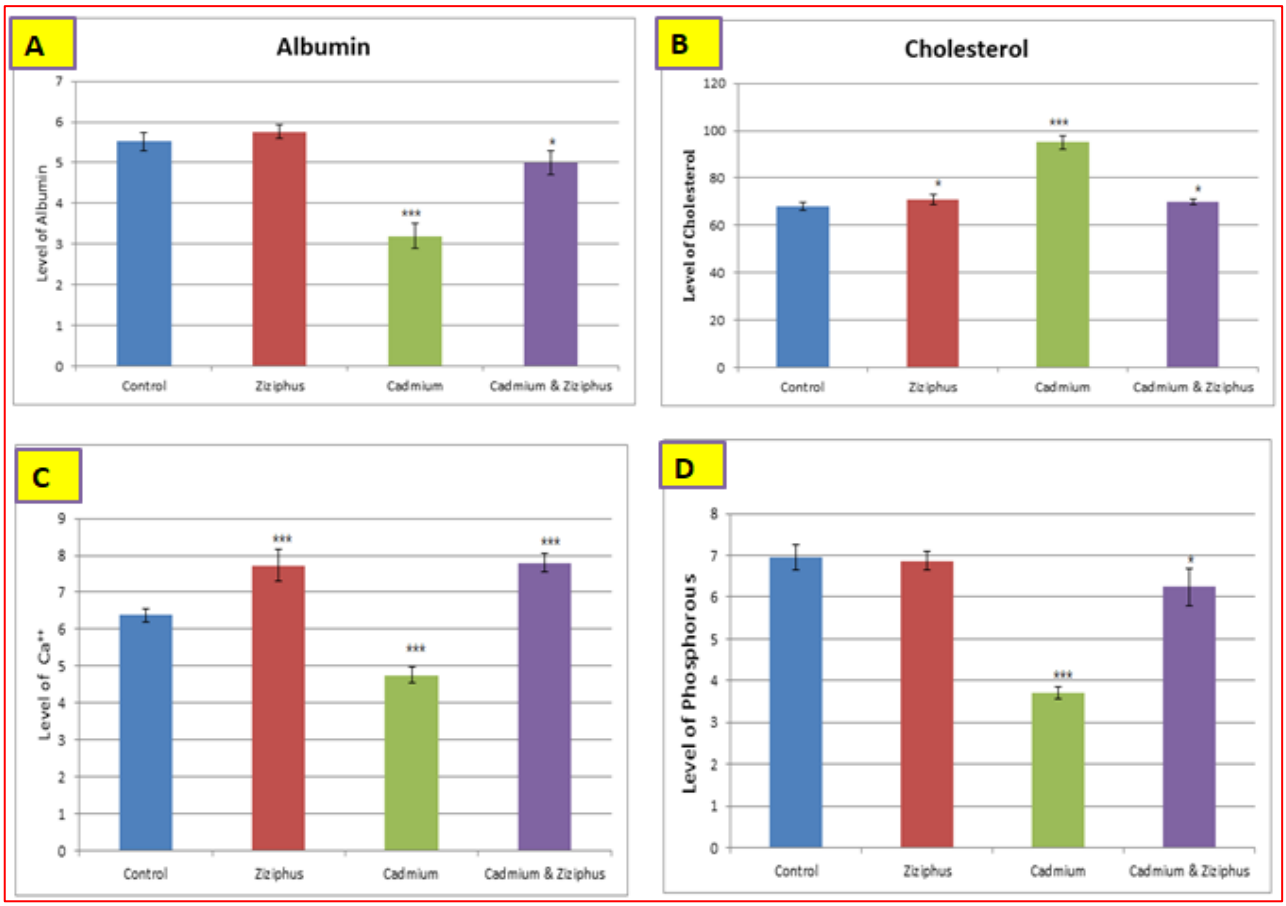

Fig.3: **A**: Showing the levels of albumin (g/dL)**, B:** the levels of cholesterol (mg/dL), **C**: the levels of calcium ions (mg/dL), and **D:** the levels of phosphorus ions (mg/dL) among the different studied groups. Note a highly significant decrease (***) in albumin, $Ca^{++,}$ and PO^{-4} but a significant increase in cholesterol for the cadmium group if compared with control. In the cadmium and *Ziziphus* group, the levels of serum albumin, cholesterol, Ca^{++}, and PO^{-4} are restored near to the normal levels as in control. ***(***highly significant, **moderately significant, * low significant)***

5. Changes in tissue SOD, GSH, and MDA

The thyroid, renal and testicular tissues from $CdCl_2$-induced rats showed a highly significant decrease (P<0.001) in the levels of SOD and GSH however these levels were restored near to the normal value after treatment with *Ziziphus* extract, especially in the renal tissues but still show a low significant decrease with control for thyroid and testes (figure4A&B).

The thyroid, kidney, and testicular tissues of the $CdCl_2$-treated group revealed a highly significant increase in the levels of MDA (P<0.001) if compared with the control. After treatment of $CdCl_2$ rats with *Ziziphus* extract, the levels of MDA in renal and testicular tissues were restored to the normal as control but for thyroid tissue, a low significant increase (P<0.05) was still noticed if compared with control (figure4C)

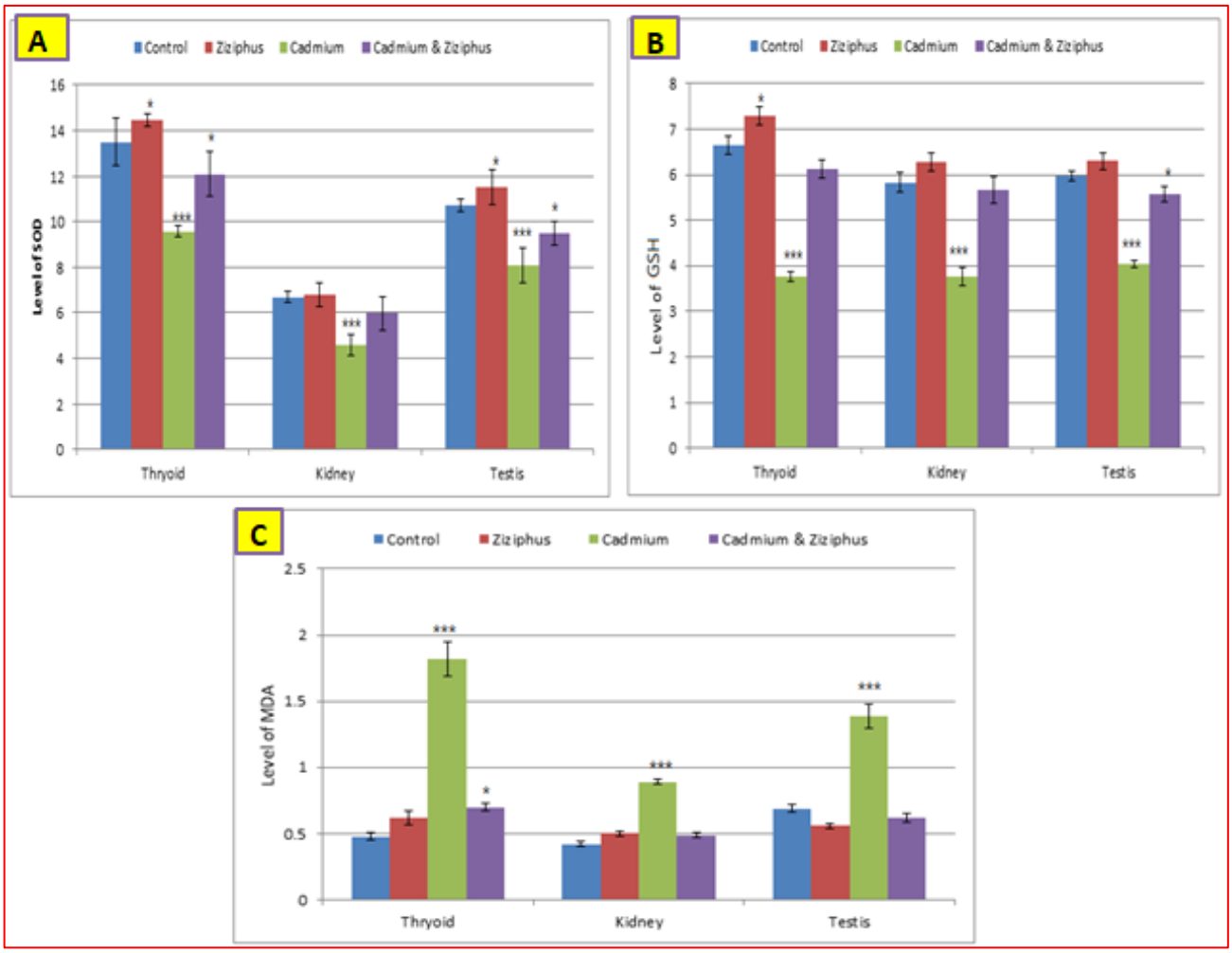

Fig.4: **A**: Showing the levels of SOD (U/mg protein)**, B:** the levels of GSH (µg/mg protein **C**: the levels of MDA (nmol//g protein) in the thyroid, kidney, and testicular tissues among the different studied groups. Note a highly significant decrease (***) SOD and GSH but a significant increase in MDA for the cadmium group if compared with control. In the cadmium and *Ziziphus* group, the levels of tissues SOD, GSH, and MDA are restored to some extent near to the normal levels as in control.***(****highly significant,* ***moderately significant,* * *low significant)***

6. Histological changes

6.1. In Thyroid tissues

The thyroid sections from the control and *Ziziphus* groups showed normal histological architecture, with cuboidal to low columnar epithelium lining small and medium-sized follicles, and the lower epithelium lining large follicles with intact basal lamina. The inter-follicular spaces contained connective tissue with blood capillaries and individual or clustered para-follicular cells. A normal secreted and light-stained colloid was found near the apical border of the cells. The inter-follicular spaces were normally formed (figure5A&B).

In $CdCl_2$ treated rats, the thyroid follicles appeared disorganized and vacuolated with remarkable cellular atrophy and pyknotic nuclei. The accumulated colloid was noticed in some areas of the section. Dead tissue is also evident in the inter-follicular space. Moreover, the follicular size was atrophied (Figure 5C). In the cadmium- *Ziziphus* group, a remarkable amelioration was noticed in the architecture of thyroid follicles whereas, the follicles appeared intact with normal size despite little vacuoles still present in the section (figure 5D).

6.2. In kidney

In the control and *Ziziphus* groups, the renal cortical sections showed normal architecture of renal tubules and renal corpuscles. The renal corpuscle consists of a glomerulus (tufts of blood capillaries) that are surrounded by Bowman's space and intact Bowman's capsule. The renal cortical tubules included well-distributed and organized proximal, distal, and collecting tubules (figure6 A&B). In $CdCl_2$exposed rats, the renal cortex showed atrophied glomeruli, tubular degenerated cells with pyknotic nuclei, especially in the proximal and collecting tubules. Other cortical tubules showed cellular hypertrophy. Additionally, hemorrhage spots among cortical tubules and glomeruli were noticed in some areas of the section (figure 6C). Co-supplementation of *Ziziphus* extract to the $CdCl_2$ group successfully restored the histological structure of the renal cortex to the normal pattern with little degenerated collecting tubules still found in the section (figure 6D).

6.3. In testes

The testicular sections from the control and *Ziziphus* groups showed normal histological structure with well-preserved seminiferous tubules. Most of the seminiferous tubules (ST) had rounded or oval contours with regular basement membrane (basal lamina). The ST contained well-organized stages of spermatogenic cells (spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids) and little spindle-shaped Sertoli cells. The sperms were seen in the lumen of the tubules. The narrow inter-tubular spaces contained clusters of Leydig cells (figure7A&B).

Rats treated with $CdCl_2$exhibited severe degenerative seminiferous tubules with a remarkable reduction in their diameter. The seminiferous tubule exhibited degenerative basal lamina and wide inter-tubular spaces with complete lysis of Leydig cells. Also, the tubular lining showed a low density of spermatogenic cells with obvious dispersed vacuoles. Furthermore, the sperms appeared few and degenerated with remarkable sloughing of the germinal epithelium of seminiferous tubules (figure7 C). In $CdCl_2$and *Ziziphus* extract-treated rats; the ST nearly retained their normal architecture. They revealed regularly rounded contours and were lined by stratified germinal epithelium showing several types of spermatogenic cells. In addition, most lumina contained a high density of sperms. The interstitial spaces contained clusters of Leydig cells (figure7 D).

7. Flow cytometric analysis of caspase-3 and annexin-v in thyroid and testicular tissues

7.1. Caspase-3

In the control group, the mean % value of positively caspase-3 expressed thyroid and testicular cells appeared in the normal range (20.9%, 23.2% respectively), however, this value was significantly elevated in $CdCl_2$ treated rats (57.8%, 57.3% respectively). On the other hand, with co-supplementation of *Ziziphu*s extract to $CdCl_2$ induced group, the mean % values of caspase-3 expressed cells of the thyroid and testicular tissues were significantly lowered (36.4%, 29.9% respectively) than that of the $CdCl_2$-exposed group alone but still significantly higher than control (figure 8).

7.2. Annexin-v

In control rats, the mean % value of thyroid and testicular viable cells (LL:82.1% and 85.9%

respectively; annexin-negative/PI-negative), the mean % value of late apoptotic cells (UR:1.8% &0.9% respectively; annexin-positive/ PI-positive), the mean % value of early apoptotic cells (LR: 6.2%&4.2% respectively; annexin-positive/PI-negative) and the % of necrotic cells (UL:9.8% &9.1%; annexin-negative/PI-positive) appeared in the normal range. On the other hand, the thyroid and testicular cells from $CdCl_2$-induced rats revealed a highly significant decrease in the mean % value of viable cells (49.8%, 58.2% respectively) and a highly significant increase in the late apoptotic cells (11.4%%, 7.3% respectively), early apoptotic cells (13.1%,6.8% respectively) and necrotic cells (25.7%,27.7% respectively) if compared with control. In $CdCl_2$-treated rats co-supplemented with *Ziziphu*s extract, a highly significant increase was recorded in the % of thyroid and testicular viable cells (72.1%, 81.8% respectively) and a highly significant decrease in the late apoptotic cells (5.6%, 3.8% respectively), early apoptotic cells (10.9%, 7.4% respectively) and necrotic cells (11.4%, 7% respectively) if compared with $CdCl_2$-induced group alone but not reach to the standard value as control (Figure 9).

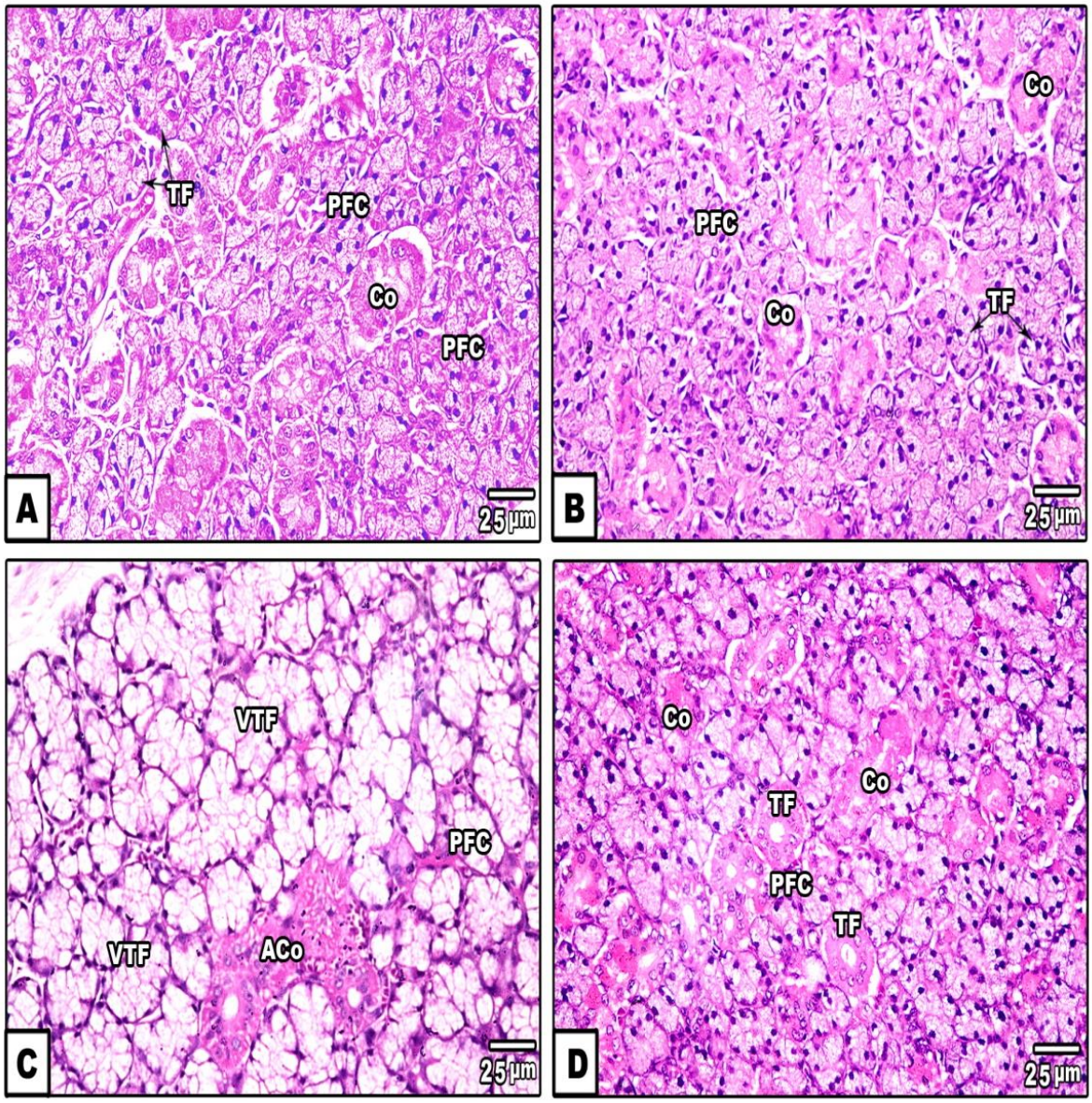

Fig 5. Photomicrograph of histological sections through the thyroid gland of control (A), *ziziphus* (B), cadmium (C), and cadmium &*zizuphus* (D) groups. Note: In images A&B the thyroid sections appear with normal histological architecture. In image C, the thyroid follicle appeared atrophied and vacuolated with accumulated colloid and pyknotic cells. In D, the thyroid follicle shows remarkable amelioration in its histological design. H&E X: 400

Abbreviations: ACo; Accumulated colloid, Co; colloid, PFC; Para follicle cells, TF; thyroid follicle and VTF; vacuolated thyroid follicle

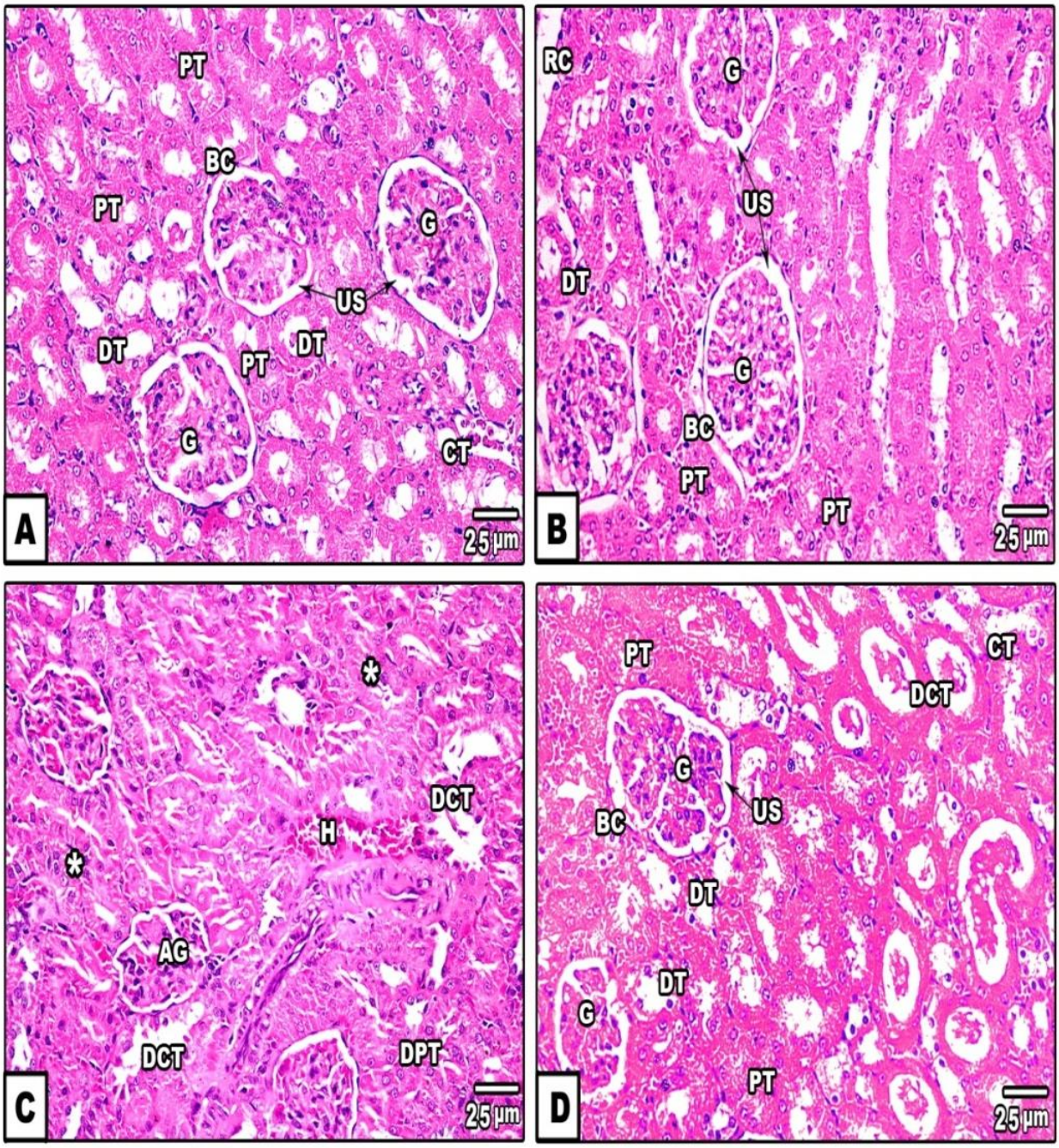

Fig.6: Photomicrograph of histological sections through the renal cortex of control **(A)**, *ziziphus***(B)**, cadmium **(C)** and cadmium &*zizuphus***(D)** groups. Note: In images A&B, the renal corpuscles & tubules appeared normal. In image **C**, the renal cortex shows atrophied glomeruli, damaged and hypertrophied tubular cells (asterisks), and inter-tubular hemorrhage spots. In image **D**, the renal corpuscles and tubules are restored to some extent to the normal architecture. H&E X: 400

Abbreviations: AG; Atrophied glomeruli, BC; Bowman's capsule, CT; Collecting tubules, DCT; Degenerated collecting tubules, DPT; Degenerated proximal tubules, DT; Distal tubules, G; Glomeruli, H; hemorrhage, PT; Proximal tubules, RC; Renal capsule, US; Urinary space.

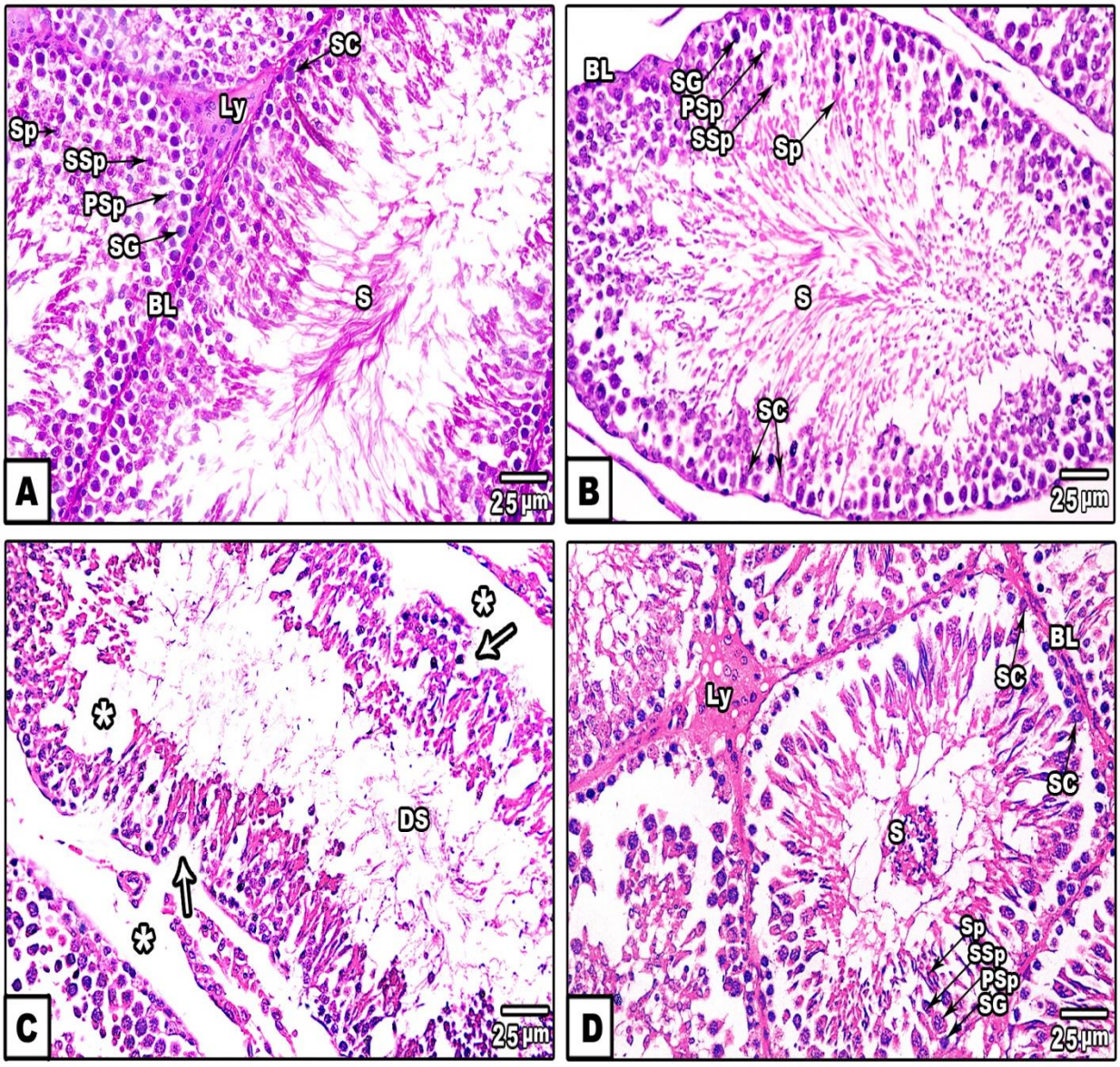

Fig.7: Photomicrograph of histological sections through the testis of control **(A)**, ziziphus **(B)**, cadmium **(C),** and cadmium &zizuphus **(D)** groups. Note: In images **A&B**, the seminiferous tubules appear preserved with obvious regulatory spermatogenic cells. In image **C**, the seminiferous tubule showed severe damage in the basal lamina (arrows), vacuolated area (star), little and degenerated spermatogenic cells, and wide inter tubular space as well as few & degenerated sperms. In image **D**, the seminefourus tubules appear with normal histological **architecture as control.** **H&E** X: 400

Abbreviations: BL; Basal lamina, DS; Degenerated sperms, LY; Leydig cell, PSp; Primary spermatocyte, S; spermatozoa, SC; Sertoli cells, SG; Spermatogonia, Sp; Spermatids, SSp; secondary spermatocyte.

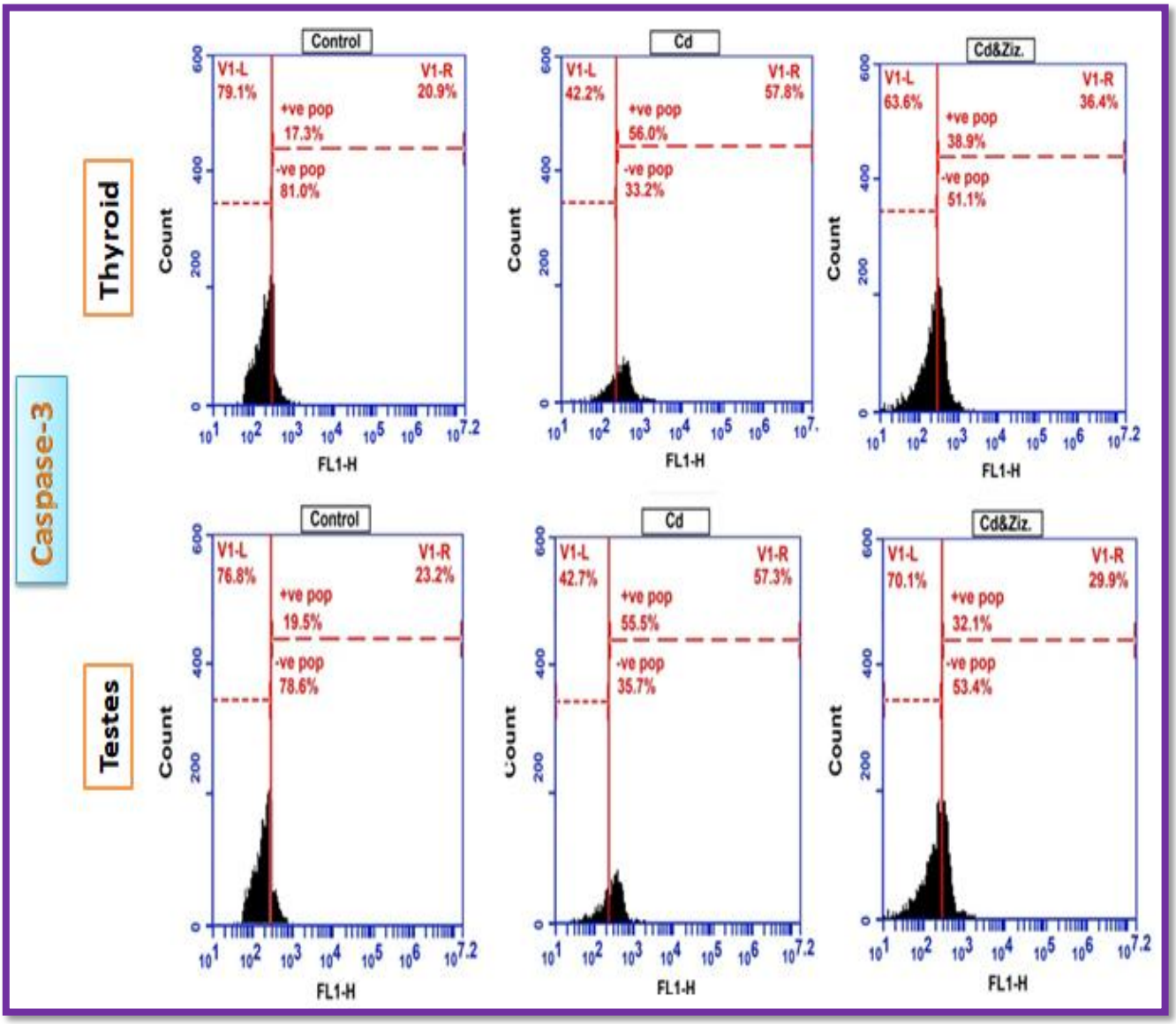

Fig.8: A flow cytometric chart illustrating the mean % value of positively expressed caspase-3 cells in the thyroid and testicular tissues among different studied groups. **Note:** In the control group, the mean % value of positively caspase-3 expressed thyroid and testicular cells appeared in the normal range (20.9%, 23.2% respectively), however, this value was significantly elevated in cadmium treated group (57.8%, 57.3% respectively). Co-supplementation of *Ziziphu*s extract to Cd-induced groups, the mean percentage values of caspase-3 expressed thyroid and testicular cells were significantly lowered (36.4%, 29.9% respectively) than that of the Cd-exposed group alone but still significantly higher than control

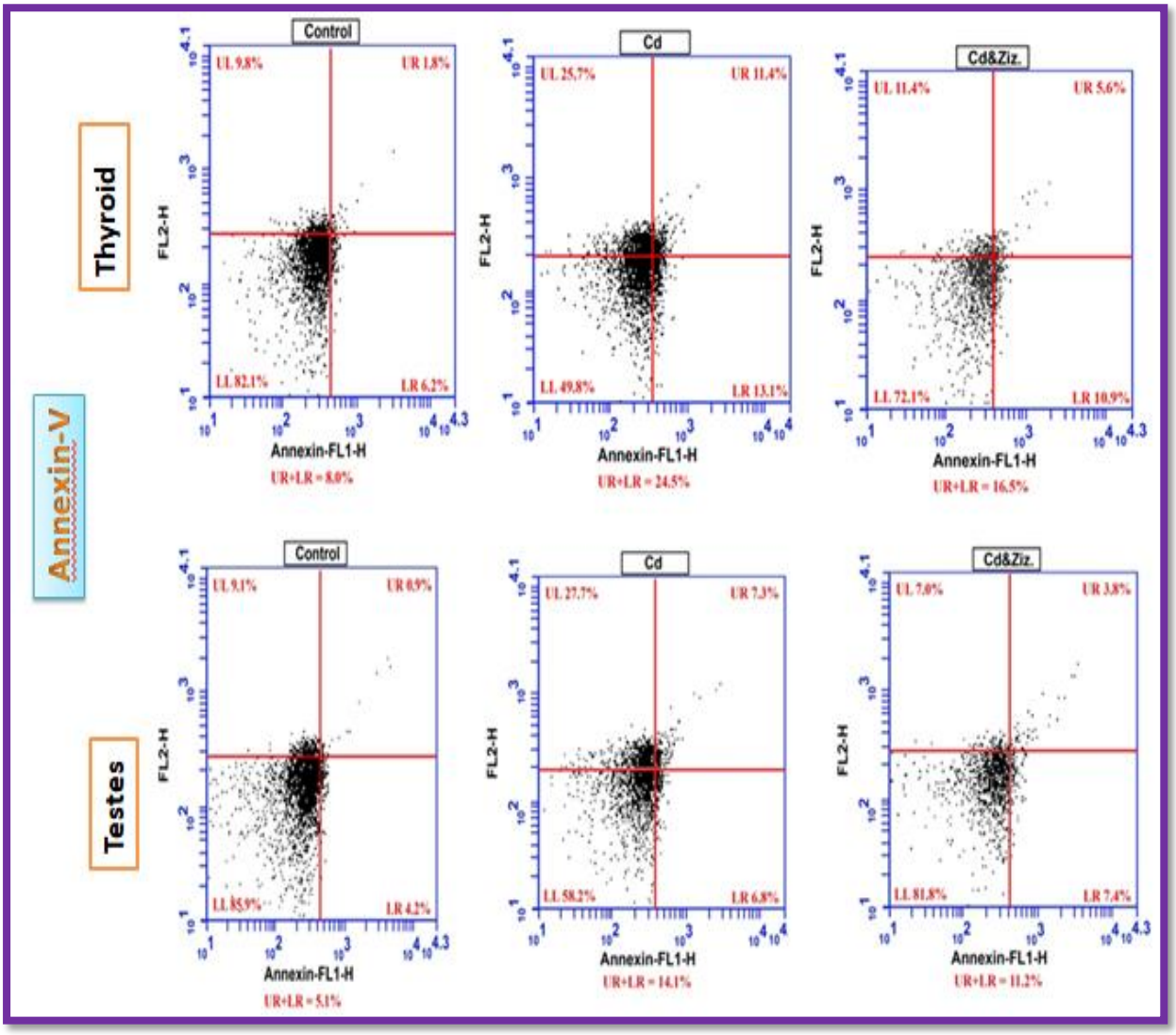

Fig.9: A flow cytometric chart illustrating the mean % of apoptosis and necrosis by FAC Scan analysis via Annexin V- FL1 H/PI in the thyroid and testicular tissues among the different studied groups of rats. Cells in the lower left quadrant indicate the % of Annexin-negative/PI-negative **(viable cells**). Cells in the lower right quadrant indicate the % of Annexin-positive/PI-negative (**early apoptotic cells).** Cells in the upper left quadrant indicate the % of Annexin-negative/PI-positive (**necrotic cells**). Cells in the upper right quadrant indicate the % of Annexin-positive/ PI-positive (**late apoptotic cells)**. **Note:** a high % value of late and early apoptotic cells in the thyroid and testicular tissues of Cd-treated rats (24.5%, 14.1 %respectively) if compared with control (8.0%, 5.1 respectively). The early and late apoptotic cells in the thyroid and testicular tissues of Cd-treated rats co-supplemented with *Ziziphus* extract appear with very low % (16.5%, 11.2 %respectively) if compared with Cd-group but do not reach the normal value as in control.

DISCUSSION

Prolonged exposure to cadmium is established with endocrine-disrupting activities (**Pilet et al., 2004; Kim et al., 2018**). Some natural plants have a powerful vital role in the amelioration of human body health. Several studies have reported that medicinal plants contain a wide variety of natural antioxidants such as phenolic acid flavonoids and tannins which possess more potent antioxidant activity **(Thirumalai et al., 2011**). ***Ziziphus spina-christi*** is one of the most useful medicinal plants which is used widely for the treatment or alleviation of several diseases. *Ziziphus* fruit extract has powerful medicinal properties like anti-inflammatory, antibacterial, analgesic, anti-neoplastic, anti-aging, and antioxidant properties **(Erenmemisoglu et al., 1995; Nisar et al., 2010).** Previous reports assured that *Ziziphus* fruits are rich in several antioxidants that are mainly attributed to their high vital contents of useful phytochemical ingredients (**Waggas and Al-Hasani 2010; Abalaka et al., 2011).** Accordingly, the present work was mainly designed to evaluate the ameliorative role of *Ziziphus spina-christi* fruit extract against $CdCl_2$-induced toxicity in thyroid, kidneys, and testes of male albino rats.

The obtained results revealed that the mean body weights of $CdCl_2$ treated rats were significantly lower than those of control however co-supplementation of *Ziziphus* fruit extract to $CdCl_2$ treated rats successfully restored the body weight near the normal. The obtained results are in agreement with previous studies **(Djebli et al., 2004 & Missoun et al., 2010**). **Sddik et al. (2010**) proved that toxic ions are one of the factors which cause body weight loss through the mechanism involving a disturbance in metabolic enzymes. Another study revealed that exposure to Cd leads to disorders in intestinal absorption and consequently body weight loss **(Graef, 1994).** The data concerned with the modulatory role of *Ziziphus* fruit extract in the maintenance of body weight go parallel with the finding of **Abalaka et al (2011)** who reported that regular supplementation of *Ziziphus* fruit extract allows for regular assimilation of all body nutrients that maintain the growth rate.

It had been reported that cadmium can alter thyroid function in experimental animals **(Lafuente et al., 2003; Hammouda et al 2008**) and humans (**Iijima et al., 2007**). **Yoshizuka et al. (1991)** suggested that Cd accumulated in the thyroid follicular epithelial cells might disturb oxidative phosphorylation, lower energy supply, and therefore inhibit the synthesis and release of thyroid hormones. Similarly, **Pilat Marcinkiewicz et al. (2003**) observed a dose-dependent effect on the structure and function of thyroid follicular cells in rats. In the current work, the declined concentration of serum T3 in cadmium-exposed rats might be due to decreased rate of transformation from T4 to T3 caused by inhibition of type-I iodothyronine 5'-monodeiodinase activity(5'-D), being a seleno-enzyme containing a selenocysteine residue as its active site (**Chaurasia et al., 1996; Shyam et al., 1997).** Cd can inhibit 5'-D activity through binding to sulfhydryl groups of this enzyme. Moreover, the decreased level of T4 in serum is mainly due to the direct damage effect of cadmium on the structure of follicular cells of the thyroid (**Yousif and Ahmed, 2009)**. The lack of significant response of TSH to decreased serum T4 and T3 levels may suggest Cd interference with pituitary regulation of thyroid hormone production and secretion **(Pavia et al., 1997).**

The data of the present study revealed a remarkable decrease in the levels of serum FSH, LH, and testosterone in the $CdCl_2$-exposed rats. **Fatma et al. (2009)** reported that LH acts upon the Leydig cells and is responsible for the production of testosterone, an androgen that exerts both endocrine activity and intra-testicular activity on spermatogenesis. It had been reported that cadmium administration significantly increased nitric oxide production leading to a decrease in testosterone synthesis in the Leydig cells through acting on the pituitary gland and inhibiting LH secretion (**Dobashi et al., 2001; Waisberg et al., 2003)**. Our findings agree with **Piasek and Laskey, (1994)** who have demonstrated obvious lowering in steroidogenesis in Cd-induced rats. Further evidence was derived from a study by **Murugesan et al., (2007)** where poor pituitary LH secretion with reduced Leydig

cell steroidogenesis was reported in a highly contaminated environment.

Concerning the obtained biochemical analysis, the albumin, calcium, and phosphorus levels showed a highly significant decrease but significant increase in cholesterol in all CdCl2 received. **Henery (2001)** recorded hypothyroidism associated with low serum albumin. Furthermore, Cd exposure increased excretion of both low and high molecular weight proteins by increasing the urinary concentrations of low molecular weight proteins (β 2 microglobulins) and induced defect in reabsorption by proximal tubules leading to decreased serum albumin **(Jarup et al., 1993). Fassett et al. (2011)** recorded that; albumin in the urine can be elevated as a result of both glomerular and tubular dysfunction and is often used in screening for early renal damage induced by chronic cadmium exposure. Furthermore, in the final stages of cadmium nephropathy, the wasting of calcium (hypercalcemia) and phosphate (hyperphosphaturia) is appeared **(Jarup et al. 2000).** Other reports suggested that cadmium can disrupt the parathyroid hormone or affects vitamin D activation in the kidney resulting decreased calcium absorption in the gut, impaired reabsorption of calcium in the renal tubules and thus increased urinary calcium excretion (**Nawrot et al., 2010; Nordberg** et **al., 2015**). Additionally, the decreased calcium levels in Cd exposed rats may be attributed to the binding of calcium with plasma protein mainly albumin, therefore calcium is in direct proportion with albumin. Cd-induced renal tubular dysfunction leads to increased urinary excretion of calcium and phosphorus (**Nordberg et al., 2015)**.

Currently, the elevated level of total cholesterol in $CdCl_2$ exposed rats goes in line with **Mohamed & El-Gharieb (2009**). **Mary (2003**) found that hypothyroidism is the most common secondary cause of high cholesterol levels in the blood.

Cadmium is known to increase oxidative stress by being a stimulant in the production of ROS, increasing lipid peroxidation, and depleting glutathione (**Gupta *et al*., 2004 and Kara *et al*., 2005)**. Cadmium also can stimulate the production of inflammatory cytokines and down-regulates the protective function of nitric oxide formation (**Navas-Acien et al. 2004**). In the present study, MDA significantly increased while the SOD and GSH were significantly decreased in all studied tissues of $CdCl_2$ treated rats. Similar observations were recorded in thyroid tissues **(Yu et al., 2018)**, renal tissues (**Murugavel** &**Pari, 2010)** and testicular tissues (**Al-Azemi et al., 2010)** exposed to cadmium. **Ghosh and Bhattacharya (1992)** suggested that Cd atoms combined with selenium atoms are excreted out of the body via bile resulting in selenium depletion. This leads reduction in GSH and increased production of ROS and hydrogen peroxide, which damage thyroid tissue.

Previous studies reported that accumulation of cadmium in testicular tissue leads to increased production of ROS and decreases various antioxidant levels. This enhances the lipid peroxidation of cell membranes, causes degeneration of seminiferous tubules, apoptosis, testicular hemorrhage, necrosis, abnormal Leydig cells, fibrosis, and reduced testicular size **(Monsefi et al., 2010**; **Alaee et al., 2014**)

Brzoska et al. (2003) revealed that Cd can interact with Zn and Cu in the renal tissues leading to a decreased level of SOD through increased production of oxygen free radicals. Moreover, Cd increased MDA concentration in the kidney indicates an escalation of lipid peroxidation in this organ due to oxidative stress. Increased peroxidation of lipid intra- and extra-cellularly explains the damage to the cells and tissues that may be attributed to the inability of antioxidant defense systems (**Karimi et al., 2012)**. Moreover, both GSH and SOD are considered to be enzymatic free-radical scavengers in cells. Thus, the decrease of both enzymes leads to an indirect increase in oxidative DNA damage and suggests that SOD plays a role in the suppression of oxygen free-radical formation and the decrease of NO generation (**Abdel-Wahhab et al., 2005).**

Low cholesterol levels in the animals treated with $CdCl_2$ in combination with *Zizyphus* extract agree with the finding of **Abdel-Wahhab et al. (2007)** who reported that *Zizyphus* extract can attenuate the

cadmium-mediated decrease in the activities of glutathione and SOD. This result indicates the anti-atherosclerotic properties of this extract and its role in the progress of cardiovascular diseases **(Morcos, 1997**). It is well documented that *Zizyphus* extract is enriched in tannin compounds which can reduce the production of ROS, and inhibition apoptosis caused by Cd **(Guerra e al., 2005**). It was also indicated that the *Zizyphus* fruits contained a high level of total phenolic especially gallic acid (**Yossef et al., 2011)** which can increase the activities of endogenous antioxidant enzymes like SOD, catalase, and GSH. Additionally, *Zizyphus* fruit extract had been demonstrated to inhibit MDA **(Xiangchun et al., 2009**).

The possible antioxidant activities of *Zizyphus* fruit extract on the testis were due to the presence of tannins (**Adzu et al., 2001)**, carotenes **(Guil-Guerrero et al., 2004),** and flavonoids **(Pawlowska et al., 2009)**. Carotenoids were found to inhibit free radicals-induced lipid peroxidation and β-carotene is one of the most efficient quenchers of singlet oxygen. It can also prevent lipid peroxidation by inhibiting the activity of lipoxygenase towards linoleate. Additionally, lycopene has a potent protective effect against ischemia-reperfusion that is induced by oxidative stress in rat testis, attributed to its ability to react with the oxygen metabolites **(Hekimoglu et al., 2009).** Therefore, it is possible that the fruit extract of *Ziziphus*, which possesses a remarkable carotenoid, attenuates cadmium-induced oxidative stress and lipid peroxidation in this study by two pathways; First, by increasing the activity of GSH and therefore rapid conversion of H_2O_2 to H_2O and preventing H_2O_2 accumulation and second, by quenching the hydroxyl radicals that trap HO leading to an oxidative breakdown of the carotenoid's molecule (**Al-Reza *et al*., 2009)**. Thus, it can be concluded that the fruit extract of *Ziziphus* may protect the membrane of testicular cells against cadmium-induced oxidative damage and appears to be a good candidate in the prevention of cadmium-induced injuries in testis. Ingestion of *Ziziphus* extracts into animals induced a significant increase in the GPx and SOD levels. Furthermore, flavanols in *Ziziphus* extract improve testicular total antioxidant capacity and its function **(Juan *et al*., 2005; Taati et al., 2011).**

In the current work, the thyroid follicles of Cd-induced rats appeared vacuolated with remarkable cellular atrophy and pyknotic nuclei as well as the follicular size was decreased if compared with control. The obtained results go parallel with the findings of previous studies (**Yousif and Ahmed, 2009; Jancic and Stosic, 2014). Ruze et al. (1999)** explained that selenium deficiency caused by cadmium treatment may lead to histological changes by activation fibrotic process in which inflammatory reaction and excess transforming growth factor β play a role in thyroid gland morphology.

Previous reports approved pronounced histological changes in the renal tissues under the Cd effect (**Smalinskienel et al., 2006; Nakazato et al., 2007**). In the current work, damage to the glomerulus and degenerative changes in the tubular lining, hypertrophy of epithelial cells, and vascular congestion were seen in some areas of the renal cortex from a group induced with $CdCl_2$. Cadmium intoxication has been reported to cause oxidative stress and decreased intracellular levels of GSH (**Ibraheem et al., 2016**). It has been suggested that Cd disturbs membranes integrity (**Nordberg et al., 1994)** generates reactive oxygen species **(El-sharaky et al., 2007),** and involves cytotoxic and inflammatory mediators (**Kayama et al., 1995)** in the kidney.

In the current study, deleterious histopathological signs were recorded in the testes of $CdCl_2$-treated rats like degenerated seminiferous tubules, damaged basal lamina, relatively wide inter-tubular spaces, and complete lysis of Leydig cells. Also, lytic and necrotic spermatogenic cells and a low density of sperms have appeared within the tubules. **Amara et al. (2008)** revealed that the cadmium can induce vascular damage resulting in decreased blood supply to the testis and decreased utilization of Zn by spermatogenic cells due to the competitive action of Cd. Further reports suggested that cadmium enters the seminiferous tubules through the blood-testis barrier and causes

focal testicular necrosis and dystrophy with a consequent reduction in germ cell numbers, leading to infertility (**Thompson** & **Bannigan**, **2008; Monsefi et al., 2010**). Additionally, different cell populations within the testis can be considered targets of cadmium toxicity (**Yang et al., 2006**), and cadmium can accumulate in germinal cells such as spermatogonia, spermatocytes, spermatid, and spermatozoa (**Aoyagi** et **al., 2002). Haouem et al. (2008)** recorded that prolonged Cd exposure can induce apoptosis of sperms.

In the current study, the histopathological signs induced by cadmium in the target tissues (thyroid, kidney, and testes) disappeared in rats treated with cadmium in combination with *Ziziphus* fruit. **Al-Sieni, (2014**) reported that the ameliorative role of *Ziziphus* fruit extract against these pathologic changes is mainly attributed to its higher content of antioxidants that help to prevent cell damage.

Activated caspase-3 is one of the key executioners of apoptosis, as it is either partially or responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly polymerase **(Cucina et al., 2008; McIlwain et al., 2013).** Currently, the obtained flow cytometric data revealed that positively activated caspase-3 cells in the thyroid and testicular tissues of cadmium-induced rats were significantly elevated if compared to control. Such findings go parallel with the results of **Haouem et al. (2008)** on testes and **Buha et al. (2018)** on thyroid induced by cadmium. **Wang et al. (2012)** reported that acute Cd toxicity is associated with testicular apoptosis through induction of oxidative stress and consequently activates caspase-3. In mammals, activated caspase-3 can cleave vital intracellular proteins. Other studies suggested that cadmium causes apoptosis and necrosis of all testicular tissue leading to disturbance of spermatogenesis, reducing sperm's motility, and finally leading to infertility (**Zhang et al., 2012; Alaee et al., 2014**). Furthermore, cadmium can induce a cascade of inflammatory reactions with increased production of pro-inflammatory cytokines, particularly TNFα which is responsible for further testicular tissue injury **(Al-Azemi et al., 2010; De Freitas et al., 2012**). Other related studies approved that cadmium is a pro-apoptotic through activation of caspase-3, depending on the conditions. For instance, in colorectal carcinoma cells (**Souza et al., 1999**), Molt-4 cells **(Hamatake et al., 2000**), and human prostate cells **(Liang et al., 1999**). Additionally, cadmium is considered an effective inducer of apoptosis in vitro in a variety of human or rodent cell lines **(Hart et al., 1999**) and in vivo exposure (**Habeebu et al., 1998).**

In the present work, FAC Scan analysis via Annexin V-FITC staining showed a significant increase in early and late positive apoptotic cells for both the thyroid and testicular tissues of cadmium-exposed rats. Further results showed a significant elevation of positive necrotic cells for the same tissues. No studies were applied to our studied tissues, but a similar finding was recorded on cadmium-induced apoptosis and necrosis in human breast (**Asara et al., 2013**)

Ziziphus fruit extract was effective in the alleviation of Cd-induced apoptosis, and necrosis and suppressed Cd-induced caspase-3 expression in the thyroid and testicular tissues of rats. The reduced caspase-3 activity observed with *Ziziphus* treatment may be due to its free radical scavenging activity and anti-inflammatory action. A similar observation was recorded by **Farmani et al. (2016).** However, our data exhibit that the anti-apoptotic effect of *ziziphus* extract is mainly attributed to the possible proliferative bioactive compounds which require isolation and further characterization.

Conclusion: Based on our findings, *Zizyphus spina-christi* extract has a powerful alleviating role against the deleterious biochemical, histopathological, and apoptotic effects in thyroid, kidneys, and testes of $CdCl_2$ exposed rats. Such amelioration is mainly attributed to the vital phytochemical constituents of this extract which have antioxidant, anti-inflammatory, and anti-apoptotic properties.

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