

# Protective and curative role of *Citrus sinensis* peel on cadmium-induced testicular and spermatogenic damage: a morphometric and immunohistochemical evaluation using monoclonal antibodies against Ki-67 and proliferating cell nuclear antigen

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## SUMMARY

This study examined the protective and curative effects of aqueous zest extract of *Citrus sinensis* on Cadmium-induced testicular tumor in animal models. Twenty four male Wistar rats (10 to 12 weeks old) weighing 165-275 g were divided into group A (treated orally with 2.5 ml/kg body weight/daily of normal saline), Group B (treated intraperitoneally with a single dose of 5mg/kg of cadmium), group C (Treated intraperitoneally with 5 mg/kg of cadmium before 10 mg/kg aqueous zest extract of *Citrus sinensis* orally), group D (treated with 5mg/kg of cadmium before 40 mg/kg extract), group E (treated with 10 mg/kg extract before 5 mg/kg of cadmium) and group F (treated with 40 mg/kg extract before 5mg/kg of cadmium). The procedure lasted for 8 weeks. Group B rats showed a significant ( $p < 0.05$ ) decrease in testis weight, testis volume, sperm count ( $p > 0.001$ ), sperm motility ( $p > 0.001$ ), abnormal sperm morphology ( $p < 0.001$ )

and a significant decrease in tubular diameter, length ( $p < 0.05$ ), cross sectional area, width, germinal epithelia height, numerical density ( $p < 0.01$ ), perimeter, number ( $p < 0.001$ ) and a significant increase in tubular lumen of the seminiferous tubules. Rats that were treated with cadmium without pre-treatment or post-treatment with extract showed marked degeneration and atrophied seminiferous tubules with absence of late stage germ cells. There was also a reduction in proliferative cell nuclear antigen (PCNA) materials and Ki67 positive cells in these rats. Interestingly, all these parameters were however attenuated in the groups that were pre-treated and post-treated with the extract.

Taken together therefore, it was concluded that aqueous zest extract of *Citrus sinensis* have protective and curative roles in the abatement of cadmium-induced testicular tumor and that these effects might be as a result of the antioxidant and free radical scavenging potentials of these nutraceuticals.

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## INTRODUCTION

Several reports have implicated cadmium (Cd) as a potent heavy metal carcinogen (category 1) both in experimental models (Sunderman, 1979; Hadley et al., 1979; Poirier et al., 1983) and human populations (Bako et al., 1982; Flanders, 1984).

Target sites for Cd carcinogenesis in rodents have been shown to include the testes (Akinloye et al., 2006; Monsefi et al., 2010), ventral prostate after parenteral exposure (Hadley et al., 1979; Poirier et al., 1983) and especially the liver and kidney (Morales et al., 2006; Liu et al., 2010).

For the main reason that Cd has been reported to exert its genotoxicity via the production of reactive oxygen species and by inhibiting DNA repair (EA, 2009), several antioxidants have been reported to attenuate testicular injury as a result of cadmium toxicity in the testes (Amara et al., 2008; Fouad et al., 2009; Tongliang et al., 2011).

The reproductive potential of species and their survival have been threatened by an increased industrial and environmental contamination (Tongliang et al., 2011) with heavy metals, and hence the need to protect or cure the testes of these maladies.

Citrus is one of the most important fruit crops grown in the world (Tao et al., 2007). The citrus family boasts of rich phytochemicals such as flavanones, polyphenols, anthocyanins and hydroxycinnamic acids. *Citrus sinensis* peel is often discarded after the succulent part of these fruit has been utilized. However, studies have indicated their medicinal potentials. Higher amounts of total phenolics such as limonene, hesperidin, narirutin, naringin and eriocitrin have been found in these waste when compared to the edible portions (Xu et al., 2008; Nada et al., 2014; Green et al., 2013).

The aim of this study was to evaluate in animal models the protective and curative effect of aqueous zest extract of *Citrus sinensis* (AZECS) on cadmium-induced oxidative damage by analyzing testicular tissue morphometrically, histochemically and immunohistochemically using monoclonal antibodies against Ki-67 and proliferating cell nuclear antigen.

## MATERIALS AND METHODS

### **Preparation of aqueous zest extracts of *Citrus sinensis* (AZECS)**

Orange fruit were peeled with a zester or grater. The white portion of the peel under the zest (mesocarp) was generally avoided by limiting the peeling depth (Liogier, 1988). The zest was thoroughly rinsed in distilled water and dried at room temperature for about 2 weeks. It was then reduced to a powdered form by grinding. The aqueous extraction was done as described by Saalu et al. (2011).

### **Aqueous extraction of AZECS**

Aqueous infusion was made by mixing a calculated volume of distilled water and powdered sample. The mixture was allowed to stand for 30 minutes before filtration. It was then centrifuged at about 3000xg for 5 min and the supernatant collected. The supernatant was cleared of particles by suction filtration using Whatmann no 1 Filter paper and cellulose filter paper. The extract was subsequently concentrated to dryness in vacuo at 40°C using a rotary evaporator and stored in a desiccator.

### **Chemicals and reagents**

Analytical grade cadmium chloride (GRM8016, Himedia, India) with 99% purity was obtained from the Department of Chemistry, Federal University Ndufu Alike Ikwo, Ebonyi State, Nigeria.

### **Experimental protocol**

Twenty four male Wistar rats (10 to 12 weeks old) weighing 165-275 g were secured from the animal house of the Department of Physiology, University of Nigeria, Enugu Campus. The animals were housed in well ventilated wire cages in the Animal Facility and an ethical approval was obtained from the Ethical Committee on Animal use with reference NNHREC/05/01/2008b-FWA00002458-1RB00002323. The rats were divided into six groups of four rats each. The rats in Group A served as the control group and were treated orally with 2.5 ml/kg body weight of Normal Saline (NS) /daily; Group B rats served as the positive control group and was given a single dose of 5 mg/kg body weight of Cd intraperitoneally (IARC; 1992; EA, 2009); Group C and D rats were given a single dose of 5 mg/kg body weight of Cd intraperitoneally, and then two weeks later they were treated orally with 10 and 40 mg/kg body weight of AZECS daily respectively, while Groups E and F rats were treated orally with 10 and 40 mg/kg body weight of AZECS respectively for two weeks daily before treatment with a single dose of Cd intraperitoneally. The protocol lasted for 8 weeks.

### **Animal sacrifice and sample collection**

The rats were first weighed and then sacrificed by cervical dislocation. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. The testes were excised and trimmed of all fat. The testicular weights of each animal were evaluated with an electronic analytical and precision balance (BA 210S, d=0.0001- Sartoriusen GA, Goettingen, Germany). The testes volumes were measured by water displacement method. The two testes of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation. One of the testes of each animal was fixed in Bouin's fluid for histological and morphometric analysis. Serum and the

remaining testes of each animal were stored at -25°C for subsequent biochemical assays.

#### **Determination of Epididymal sperm parameters: Spermatozoa progressive motility**

The motility score was estimated using the method described by Sonmez et al. (2005). Motility estimates were performed from three different fields in each sample, and their mean was used as the final motility score. The motile spermatozoa were classified as either rapid linear progressive or sluggish non-linear motile.

Spermatozoa in the right epididymis were counted by a modified method of Yokoi and Mayi (2004), while the normal and abnormal spermatozoa morphology was estimated as described by Atessahin et al. (2006). Spermatozoon was considered abnormal morphologically if it had rudimentary tail, round head and detached head.

#### **Tissue Preparation for Histology, Histochemistry and Immunohistochemistry**

The organs were processed for histological analysis. Prior to embedding, it was ensured that the mounted sections were orientated perpendicular ("vertical sections") to the long axes of the testes. Serial sections of 4 µm thick were obtained from a solid block of tissue, fixed on clean slides to which Mayer's egg albumin had been coated to cement the sections to the slides properly and were stained. For histological and histochemical study, sections were stained with H&E (GHS132 and HT1102128, Sigma-Aldrich) and Periodic Acid-Schiff (PAS) (395B, Sigma-Aldrich) reaction with hematoxylin counterstaining, after which they were passed through a mixture of equal concentration of xylene and alcohol. Following clearance in xylene the sections were oven-dried between 35°C and 40°C (Sheehan and Hrapchak, 1987).

For immunohistochemical study, sections of testes were deparaffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mM, 20 min). This was followed by endogenous peroxidase blocking in 3% H<sub>2</sub>O<sub>2</sub> for 10 min and incubation with rabbit antimouse Ki67 monoclonal antibody (1:300; Millipore, AB9260). The sections were incubated with their related secondary antibodies at room temperature for 1 h after washing the slides with phosphate buffered saline, followed by detection with 3-amino-9-ethylcarbazole, a chromogen. The slides were counterstained with hematoxylin (GHS132, Sigma-Aldrich) and mounted in Faramount aqueous mounting medium. After immunohistochemical detection of the Ki67 nuclear antigen, cells that stained positive for Ki67 were counted in each sample. One hundred tubules were counted in each group, and the percentage of positive cells in the tubules was determined. The slides were viewed under a research microscope (Eclipse 80i, Nikon, Japan) connected to a computer monitor for qualitative and quantitative

evaluation (Falana et al., 2013).

PCNA immunohistochemical staining was done using primary antiserum to PCNA (Clone PC 10, DAKO A/S Denmark). The primary antibody was diluted in Trisbuffered saline with a dilution of 1:50. The sections were incubated with the primary antibody overnight at +4°C. The binding of the primary antibody was observed using a commercial avidinbiotin- peroxidase detection system. A mouse monoclonal antibody was applied in place of the primary antibody to act as a negative control. Sections from the small intestine were used as a positive control. Then the slides were stained with diaminobenzene (DAB) as the chromogen and counterstained with hematoxylin.

The tissue slides were examined for PCNA immunostaining using light microscope (x 400). Microscopic fields were chosen at random. Five fields per slide and seven slides per rat were evaluated. The PCNA-LI for each seminiferous tubule was estimated as a percentage of immuno-labeled cells to all basal cells.

#### **Determination of Morphometric Parameters**

This was done with the H&E Slides. For each testis, seven "vertical sections" from the polar and the equatorial regions were sampled (Qin and Lung, 2002). Seven "vertical sections" per testis were selected by a systematic sampling method that ensured fair distribution between the polar and equatorial regions of each testis.

Diameter, perimeter, length, width, roundness, lumen diameter and germinal epithelia height of the seminiferous tubules of the testes were estimated with a digimizer software programme. Unbiased numerical estimation of the following morphometric parameters was determined using a systematic random scheme (Gundersen and Jenson, 1987): cross-sectional area of the seminiferous tubules (AC); number of profiles of seminiferous tubules per unit area of testis (NA); and numerical density of the seminiferous tubules (NV).

For each morphometric parameter, five randomly selected fields from all the seven sections of a single testis were viewed and estimation on each carried out. The average from a total of seventy readings from five fields in seven sections of the two testes of one rat was obtained, and this was recorded as one observation. Estimation of volume density of testicular components and number of seminiferous tubules were done on a computer monitor onto which a graph sheet was superimposed and on which slides were projected from a research light microscope (Eclipse 80i, Nikon, Japan).

#### **Cross-sectional area (AC) of the seminiferous tubules of the testes**

The cross-sectional areas of the seminiferous tubules were determined from the formula  $AC = \pi D^2/4$ , (where  $\pi$  is equivalent to 3.142 and D the

mean diameter of the seminiferous tubules).

### Number of profiles of seminiferous tubules in a unit area of testis (NA)

The number of profiles of seminiferous tubules per unit area was determined by using the unbiased counting frame proposed by Gundersen (1977).

### Numerical Density (NV) of seminiferous tubules

This was determined with the modified Floderus equation:

$NV = NA / (D + T)$  (Gilliland et al., 2001) where, NA is the number of profiles per unit area, D is the diameter and T the average thickness of the section.

### Statistical Analysis

All data were expressed as mean  $\pm$  SD of number of experiments ( $n = 4$ ). The level of homogeneity among the groups was tested using Analysis of Variance (ANOVA), as done by Snedecor and Cochran (1980) with Scheffe's post hoc test. The level of significance was considered at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ .

Analysis of data was done using both electronic calculator and Statistical Package for Social Sciences (SPSS)/ PC computer program (version 20.0 SPSS, Cary, NC, USA).

## RESULTS

### Testicular weight and Volume

This study observed a significant decrease in testis weight ( $p < 0.01$ ) and volume ( $p < 0.05$ ) of rat

exposed to Cd without pre-treatment or post-treatment with AZECS. Although there was a significant improvement in the testes weight of rat treated with the extract, the testicular volume of the entire group showed no ( $p > 0.05$ ) significant difference from the positive control (Table 1). Also, there was no significant difference between the groups treated with varying doses.

### Sperm parameters

#### Sperm motility and count in Wistar rats

Table 2 shows that mean motility ( $89.2 \pm 7.6\%$ ) and sperm count ( $134.2 \pm 9.9 \times 10^6/\text{ml}$ ) for the animals treated with normal saline were normal. However, results showed a significant ( $p > 0.001$ ) reduction in sperm motility, progressivity and count in the group treated with Cd without pre-treatment or post-treatment with AZECS (Table 2).

The result of normal sperm morphology and abnormal sperm morphology in animals treated with cadmium alone were in line with that of the count and motility at  $p > 0.001$ .

With the exemption of Group E, which had a non-significant increase in sperm motility and count, but a significant ( $p > 0.001$ ) increase in normal sperm morphology and significant ( $p > 0.001$ ) decrease in abnormal sperm morphology, the other groups of rat pre-treated and post-treated with AZECS had a significant increase in sperm count, sperm motility, normal sperm morphology ( $p < 0.001$ ) and a significant ( $p > 0.001$ ) decrease in abnormal sperm morphology. However, the sperm progressivity of all the groups were not significantly

**Table 1.** The effects of Cd and AZECS on testicular weight and volume of male rats

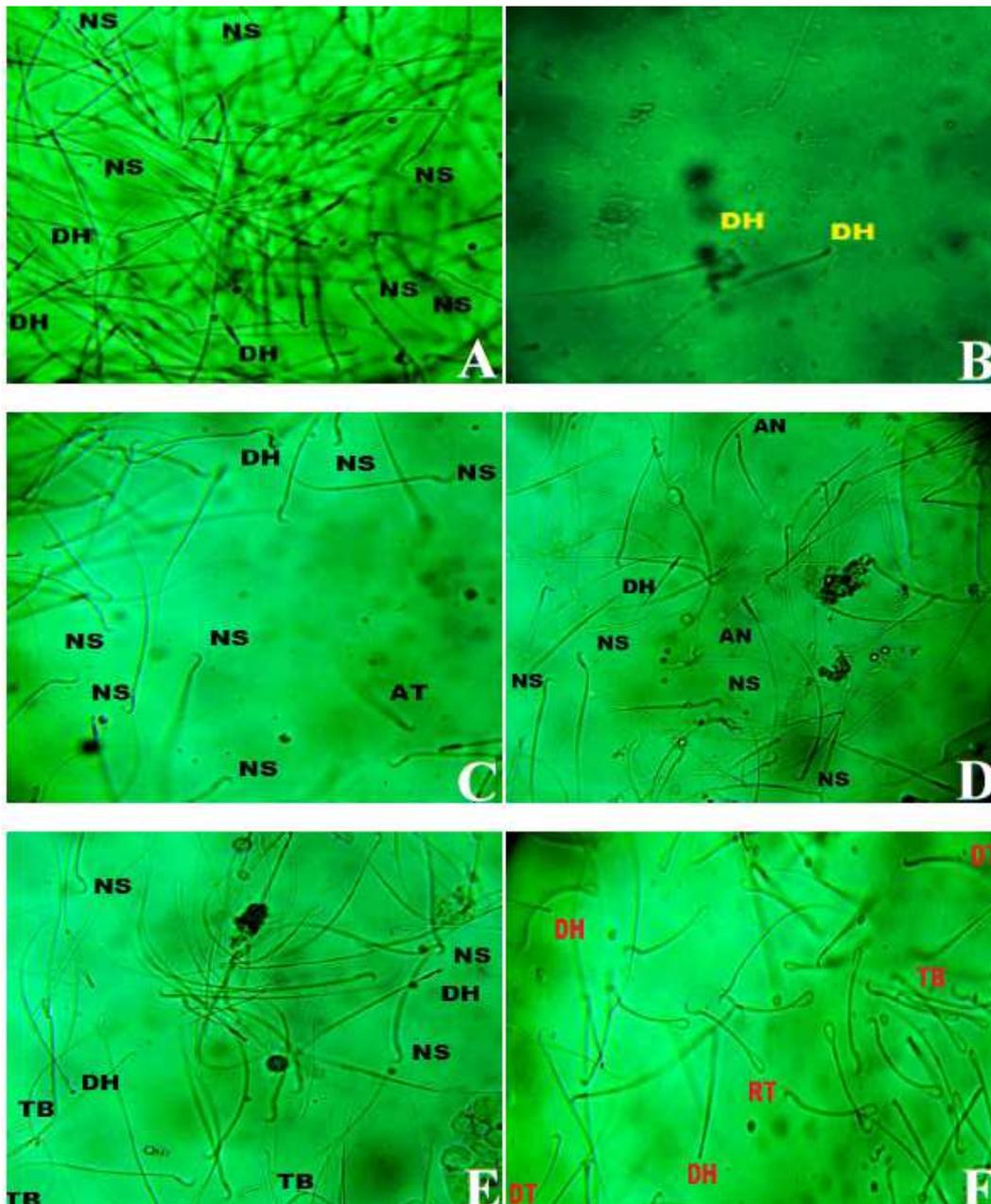
Treatment Groups	Initial Body Weight (g)	Final Body Weight (g)	Testes Weight (g)	Testes Volume (ml)	Testes Weight/ Body Weight Ratio
Group A	289.3 $\pm$ 58.5	327.8 $\pm$ 39.7	1.6 $\pm$ 0.2	1.5 $\pm$ 0.2	0.005
Group B	275.5 $\pm$ 21.0	201.6 $\pm$ 13.2	0.5 $\pm$ 0.1 <sup>b</sup>	0.6 $\pm$ 0.1 <sup>a</sup>	0.002
Group C	180.4 $\pm$ 18.2	231.5 $\pm$ 22.1	1.2 $\pm$ 0.2 <sup>3</sup>	0.9 $\pm$ 0.1	0.005
Group D	165.4 $\pm$ 10.7	215.1 $\pm$ 13.5	1.3 $\pm$ 0.1 <sup>3</sup>	0.8 $\pm$ 0.1	0.006
Group E	180.4 $\pm$ 12.6	221.1 $\pm$ 31.1	1.2 $\pm$ 0.1 <sup>3</sup>	0.5 $\pm$ 0.2	0.005
Group F	262.3 $\pm$ 48.9	259.2 $\pm$ 77.9	1.3 $\pm$ 0.1 <sup>3</sup>	0.7 $\pm$ 0.2	0.005

<sup>a,b,c</sup> represent significant increases or decreases at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively when compared to negative control (Group A). <sup>1,2,3</sup> represent significant increases or decreases at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  when compared to positive control. Values are means  $\pm$  SD.  $n = 4$ .

**Table 2.** Effects of Cd and AZECS on sperm count, motility, progressivity and morphology in Wistar rats

Treatment Groups	SPERM COUNT (x10 <sup>6</sup> /mL)	SPERM MOTILITY (%)	PROGRESSIVE MOTILITY (%)	NORMAL MORPHOLOGY (%)	ABNORMAL MORPHOLOGY (%)
GROUP A	134.2 $\pm$ 9.9	89.2 $\pm$ 7.6	a <sup>1</sup>	95.0 $\pm$ 2.9	5.0 $\pm$ 2.9
GROUP B	28.2 $\pm$ 7.3 <sup>c</sup>	17.0 $\pm$ 4.7 <sup>c</sup>	b <sup>1</sup>	14.2 $\pm$ 5.3 <sup>c</sup>	85.7 $\pm$ 5.3 <sup>c</sup>
GROUP C	83.2 $\pm$ 13.0 <sup>3</sup>	60.2 $\pm$ 6.9 <sup>3</sup>	b <sup>1</sup>	51.2 $\pm$ 6.1 <sup>3</sup>	51.2 $\pm$ 10.5 <sup>2</sup>
GROUP D	107.2 $\pm$ 10.5 <sup>3</sup>	74.2 $\pm$ 6.0 <sup>3</sup>	b <sup>1</sup>	58.2 $\pm$ 8.1 <sup>3</sup>	41.7 $\pm$ 8.1 <sup>3</sup>
GROUP E	74.7 $\pm$ 29.5	42.2 $\pm$ 28.3	b <sup>1</sup>	58.5 $\pm$ 10.4 <sup>3</sup>	41.5 $\pm$ 10.4 <sup>3</sup>
GROUP F	96.0 $\pm$ 6.03	56.3 $\pm$ 4.0 <sup>3</sup>	b <sup>1</sup>	64.6 $\pm$ 7.7 <sup>2</sup>	35.3 $\pm$ 7.7 <sup>2</sup>

<sup>a,b,c</sup> represent significant increases or decreases at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively when compared to negative control (Group A). <sup>1,2,3</sup> represent significant increases or decreases at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  when compared to positive control. Values are means  $\pm$  SD.  $n = 4$ . a<sup>1</sup> = rapid linear progressive motility, b<sup>1</sup> = show sluggish linear or non-linear motility.



**Fig. 1.** Sperm morphology of group A rat (2.5ml/kg of NS), B (5mg/kg of Cd), C (5mg/kg of Cd +10mg/kg AZECS), D (5mg/kg of Cd +40mg/kg AZECS), E (10mg/kg AZECS+5mg/kg of Cd) and F (40mg/kg AZECS+5mg/kg of Cd) for 8 weeks. RH: Round head, DH: Detached, Double tail, RT: Round tail, TB: Twisted body.

different from those of the positive control (Table 2 and Fig. 1).

#### **Testes histological profiles**

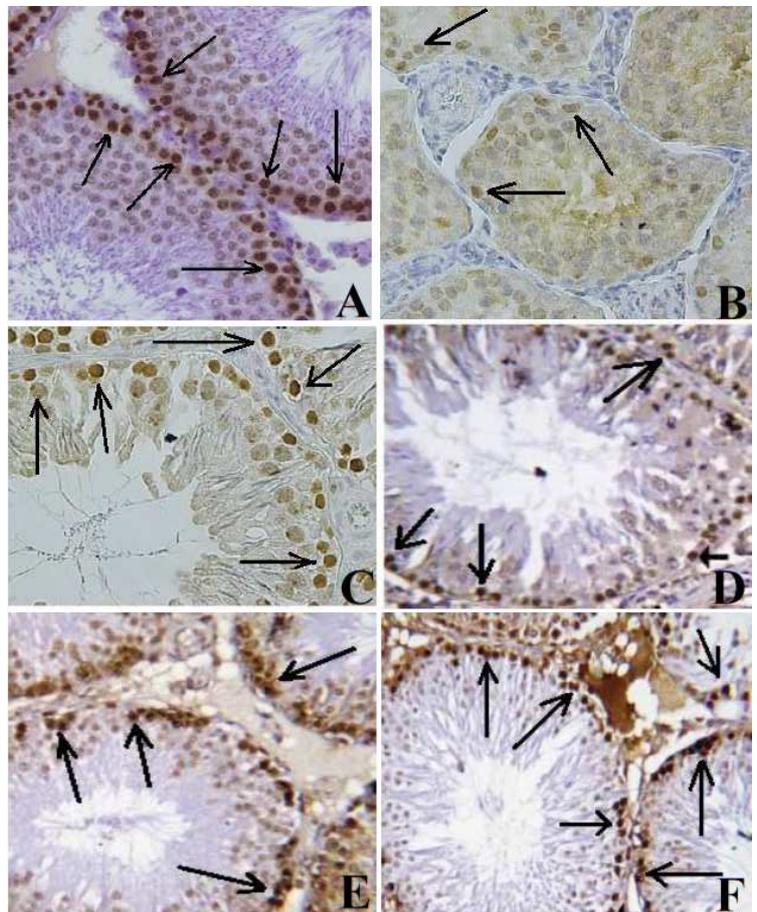
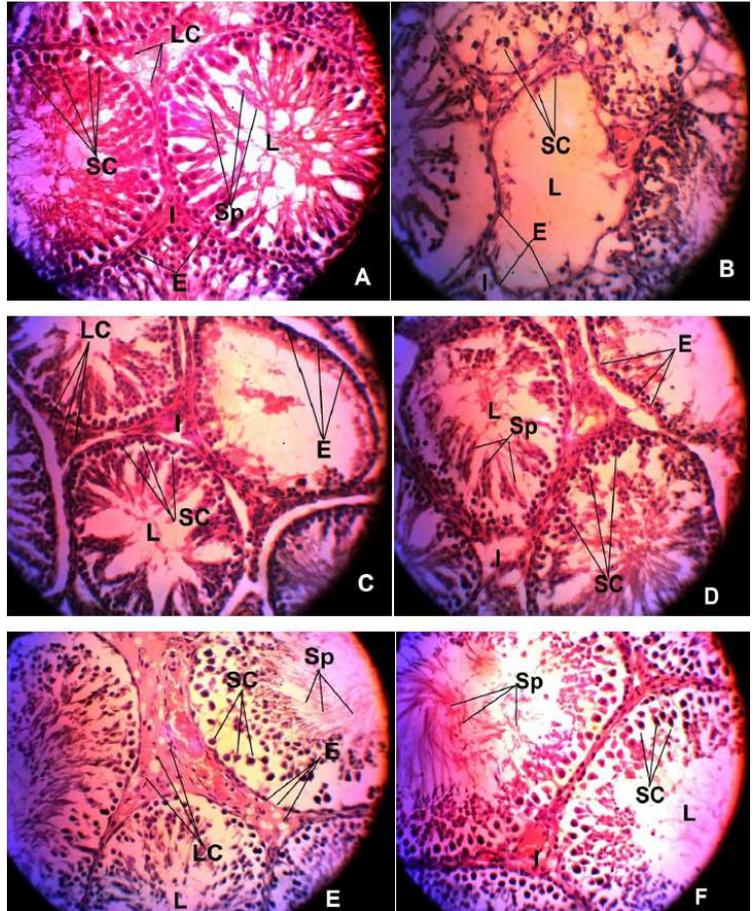
The testicular sections of control rats had normal histological profile characterized by hyperspermatozoa concentration in the lumen (Fig. 2A).

Observed in the histological profile of rat treated with Cd alone were marked degeneration and atrophied seminiferous tubules, interstitial edema, degenerated and vacuolated germinal epithelium, absence of late stage germ cells, degenerated spermatogenic cells and absence of sperm bun-

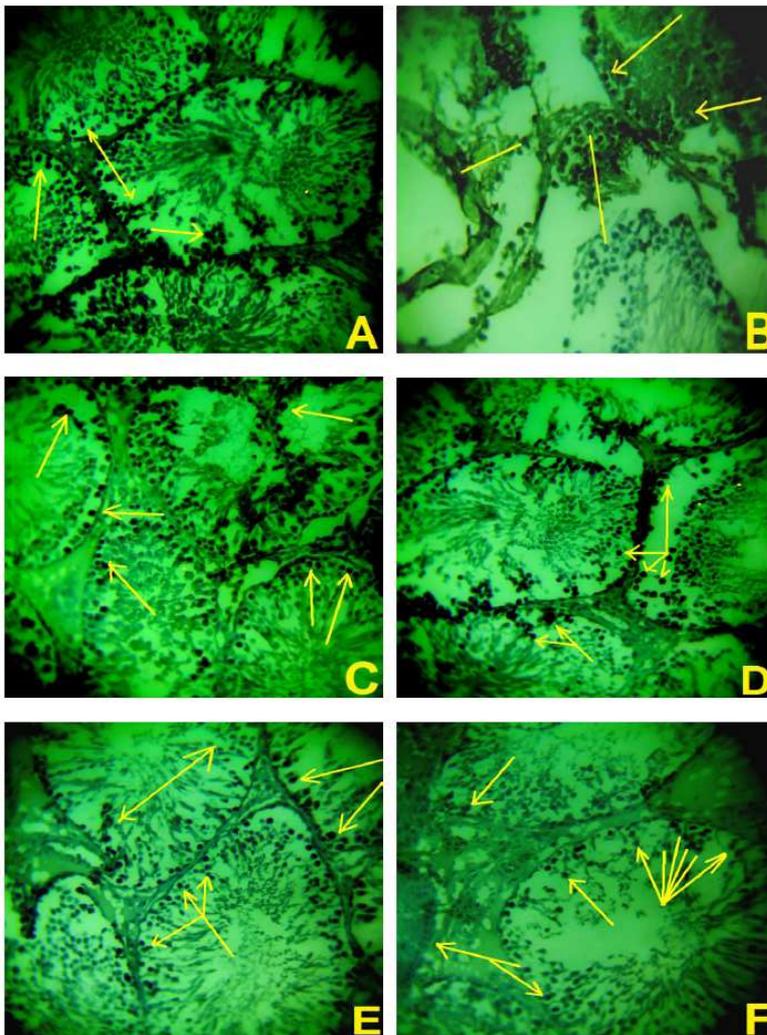
dles in most tubules when compared to those of the control that had normal histological profiles. The interstitium and Leydig cells appear to be mostly affected (Fig. 1B).

However, the groups of rats that had AZECS pretreatment and post-treatment had a significant improvement in late stage germ cells and spermatogenic cells. The Leydig cells were evident with seminiferous tubular boundary evidently preserved. There was presence of sperm bundles radiating towards the seminiferous tubular lumen in most tubules (Figs. 2C-F).

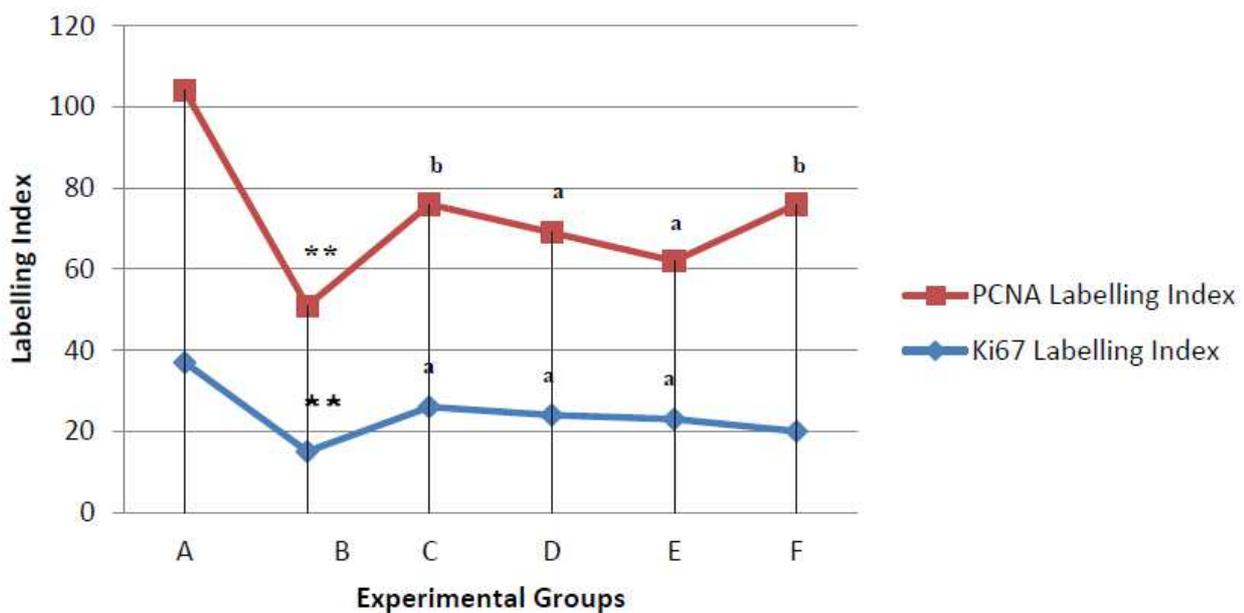
**Fig. 2.** Histological cross-sections of testis of group A (2.5ml/kg of normal saline), B (5mg/kg of Cd), C (5mg/kg of Cd +10mg/kg AZECS), D (5mg/kg of Cd +40mg/kg AZECS), E (10mg/kg AZECS+5mg/kg of Cd) and F (40mg/kg AZECS+5mg/kg of Cd) for 8 weeks. Stain: H&E. Slide showing the seminiferous tubules; L: Lumen; E: Epithelium; I: Interstitium; SC: Sertoli cells; LC: Leydig cells. Magnification: x 400.



**Fig. 3.** PCNA immunohistochemical staining: cross-section of testis of group A rat (2.5ml/kg of NS), B (5mg/kg of Cd), C (5mg/kg of Cd +10mg/kg AZECS), D (5mg/kg of Cd +40mg/kg AZECS), E (10mg/kg AZECS+5mg/kg of Cd) and F (40mg/kg AZECS+5mg/kg of Cd) for 8 weeks. Arrows showing strong immunostaining/nuclear reaction in the tubules. Magnification: x 400.



**Fig. 4.** Ki67 immunohistochemical staining: cross-section of testis of group A rat (2.5ml/kg of NS), B (5mg/kg of Cd), C (5mg/kg of Cd +10mg/kg AZECS), D (5mg/kg of Cd +40mg/kg AZECS), E (10mg/kg AZECS+5mg/kg of Cd) and F (40mg/kg AZECS+5mg/kg of Cd) for 8 weeks. Arrows show Ki67 positive reactions in the seminiferous tubules. Magnification: x 400.



**Fig. 5.** The effects of Cd and aqueous zest extract of *Citrus sinensis* on PCNA and Ki67 immunopositivity of male rats. \*, \*\* represent significant increases or decreases at  $p < 0.05$  and  $p < 0.005$  respectively when compared to negative control (Group A). a, b represent significant increases or decreases at  $p < 0.05$  when compared to positive control (Group B). Values are means  $\pm$  SD. n = 4.

**Table 3.** Effects of Cd and AZECS on diameter (D), perimeter (PST), length (LST), width (WST), roundness (RST) and lumen diameter (LD) of seminiferous tubules (LST) of Wistar rats

Treatment Groups	D (µm)	PST (µm)	LST (µm)	WST (µm)	RST (µm)	LD (µm)
GROUP A	278.5 ± 91.6	900.4 ± 121.0	342.7 ± 72.3	232.7 ± 24.6	0.84 ± 0.1	10.9 ± 5.9
GROUP B	250.9 ± 65.40 <sup>a</sup>	790.3 ± 178.4 <sup>c</sup>	328.7 ± 57.2 <sup>a</sup>	216.9 ± 55.4 <sup>b</sup>	0.87±0.0	200.2 ± 7.1 <sup>c</sup>
GROUP C	253.6 ± 24.3	761.0 ± 34.4	264.1 ± 2.2	219.2 ± 37.4 <sup>1</sup>	0.9 ± 0.0	156.6 ± 44.6 <sup>3</sup>
GROUP D	276.7 ± 2.51 <sup>1</sup>	868.7 ± 41.2 <sup>2</sup>	312.0 ± 14.9	237.6 ± 24.5 <sup>2</sup>	0.87 ± 0.0	105.4 ± 12.9 <sup>3</sup>
GROUP E	245.5 ± 16.6 <sup>a</sup>	976.1 ± 163.6 <sup>3</sup>	373.2 ± 19.5 <sup>3</sup>	249.1 ± 7.3 <sup>2</sup>	0.71 ± 0.1 <sup>a</sup>	45.8 ± 12.9 <sup>2</sup>
GROUP F	277.4 ± 41.3 <sup>1</sup>	1071.1 ± 87.5 <sup>3</sup>	388.9 ± 59.5 <sup>3</sup>	282.8 ± 40.0 <sup>3</sup>	0.81 ± 0.1	103.1 ± 29.1 <sup>3</sup>

<sup>a,b,c</sup> represent significant increases or decreases at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively when compared to negative control (Group A).  
<sup>1,2,3</sup> represent significant increases or decreases at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  when compared to positive control. Values are means ± SD. n = 4.

**Table 4.** Effects of Cd and AZECS on mean number of germinal epithelia height (GEH), cross-sectional area (Ac), number of profiles per unit area (NA) and numerical density (NV) of seminiferous tubules of Wistar rats.

Treatment Groups	GEH (µm)	Ac (x 103µm <sup>3</sup> )	NA (x10-8 µm-2)	NV (x10-10µm-2)
GROUP A	25.3 ± 2.8	23.7 ± 0.5	27.0 ± 0.4	17.6 ± 0.5
GROUP B	10.2 ± 7.1 <sup>c</sup>	15.1 ± 0.5 <sup>c</sup>	12.6 ± 0.6 <sup>c</sup>	8.3 ± 0.1 <sup>c</sup>
GROUP C	30.7 ± 0.6 <sup>3</sup>	14.8 ± 0.3	18.4 ± 0.7 <sup>3</sup>	11.2 ± 0.1 <sup>3</sup>
GROUP D	27.3 ± 3.7 <sup>3</sup>	20.5 ± 0.3 <sup>3</sup>	22.6 ± 0.5 <sup>3</sup>	18.7 ± 0.3 <sup>3</sup>
GROUP E	22.6 ± 3.7 <sup>3</sup>	16.5 ± 0.3	14.3 ± 0.2 <sup>1</sup>	8.9 ± 0.5
GROUP F	47.8 ± 3.5 <sup>3</sup>	25.3 ± 0.2 <sup>3</sup>	22.6 ± 0.5 <sup>3</sup>	12.50 ± 0.4 <sup>2</sup>

<sup>a,b,c</sup> represent significant increases or decreases at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively when compared to negative control (Group A).  
<sup>1,2,3</sup> represent significant increases or decreases at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  when compared to positive control. Values are means ± SD. n = 4.

### Testes Immunohistochemical

#### Profiles PCNA immunostaining

From our result, the total PCNA positive values of the group treated with Cd alone was significantly ( $p < 0.05$ ) different from that of the control. However, the PCNA positive cells of those groups pre-treated and post-treated with AZECS had a significant increase in positivity when compared to the corresponding control (Figs. 3 and 5).

There was no noted difference ( $p > 0.05$ ) between the groups treated with varying doses of AZECS.

#### Ki67 immunostaining

As seen in Figure 4 and 5, there was a significant ( $p < 0.05$ ) decrease in Ki67 positive cells in the group treated with Cd alone when compared to the negative control that had strong Ki67-stained cells mainly localized in the spermatogonial germ cells of the seminiferous tubules. The positivity improved ( $p < 0.05$ ) after pre-treatment and post-treatment with AZECS (Figs. 3-5).

### Testicular histo-morphometry

Diameter, perimeter, Width (WST), roundness (RST) and lumen diameter (LD), Germinal epithelia height (GEH), cross-sectional area (Ac) number of profiles per unit area (NA) and numerical density (NV), length of seminiferous tubules of Wistar rats.

The negative control group of rats had normal

mean diameter (278.5 ± 91.6 µm), perimeter (900.4 ± 121.0 µm), length (342.7 ± 72.3 µm), width (232.7 ± 24.6 µm), roundness (0.84 ± 0.1 µm), lumen diameter (10.9 ± 10.9 µm), mean number of germinal epithelia height (25.3 ± 2.8 µm), cross-sectional area (23.7 ± 0.5 (x 103µm<sup>3</sup>)), number of profiles per unit area (27.0 ± 0.4 (x10-8 µm-2)) and numerical density (17.6 ± 0.5 (x10-10µm-2)) of seminiferous tubules in negative control groups (Tables 3 and 4).

However, the group that had Cd-alone had a significant decrease in tubular diameter ( $p < 0.05$ ), perimeter ( $p < 0.001$ ), length ( $p < 0.05$ ), width ( $p < 0.01$ ), lumen diameter ( $p < 0.001$ ), germinal height ( $p < 0.001$ ), cross sectional area ( $p < 0.001$ ), number of profiles per unit area ( $p < 0.001$ ) and numerical density ( $p < 0.001$ ) (Tables 3 and 4).

Although, group E rats had a significant ( $p < 0.05$ ) reduction in tubular diameter and roundness when compared to the negative control group, the groups of rat treated with a higher dose of extract (Group D and F) had a significant increase in diameter ( $p < 0.05$ ), perimeter ( $p < 0.01$  and  $p < 0.001$  respectively), width ( $p < 0.01$  and  $p < 0.001$  respectively), decrease in lumen diameter ( $p < 0.001$ ), increase in germinal epithelium height ( $p < 0.01$  and  $p < 0.001$  respectively), cross-sectional area ( $p < 0.001$ ), number of profiles per unit area ( $p < 0.001$ ) and numerical density ( $p < 0.001$  and  $p < 0.001$  and  $p < 0.001$  respectively).

< 0.01 respectively) when compared to the positive control group.

The animals treated with cadmium before low dose AZECA had a significant increase in width ( $219.2 \pm 37.4$ ), decrease in lumen diameter ( $156.6 \pm 44.6$ ), increase in germinal height ( $30.7 \pm 0.6$ ), number of profiles per unit area ( $18.4 \pm 0.7$ ) and numerical density ( $11.2 \pm 0.1$ ) of seminiferous tubules when compared to the positive control group.

Rats treated with low dose AZECA before cadmium treatment had a significant decrease in lumen diameter ( $45.8 \pm 12.9$ ,  $p < 0.01$ ), significant increase in perimeter ( $976.1 \pm 163.6$ ,  $p < 0.001$ ), length ( $373.2 \pm 19.5$ ,  $p < 0.001$ ), width ( $249.1 \pm 7.3$ ,  $p < 0.01$ ), increase in germinal height ( $22.6 \pm 3.7$ ,  $p < 0.01$ ), number of profiles per unit area ( $14.3 \pm 0.2$ ,  $p < 0.05$ ) and a non-significant increase in numerical density ( $8.9 \pm 0.5$ ) of seminiferous tubules when compared to the positive control group.

## DISCUSSION

Cadmium is one of environmental pollutants from electroplating, fertilizers, pigment and plastic manufactures (Ognjanovic et al., 2008). Humans and animals can easily be exposed to cadmium toxicity by consuming plants, water and air. Most animals with scrotal testes are susceptible to cadmium-induced testicular toxicity (King et al., 1999). Although only about 1-2 % of acute cadmium dose is usually taken up by the testes, testicular toxicity is almost invariably evident.

In this study, we aimed to determine the role of AZECS on the testis of male Wistar rats exposed to Cd using spermiographic, morphometric, immunohistochemical and physiological approaches.

We observed a significant ( $p < 0.05$ ) decrease in testicular weight and testicular volumes of rat treated with Cd alone. Our result is in accordance with that of Rekha et al. (2011) which state that derangement in some testicular structures such as seminiferous tubules and Leydig cells could lead to about 70% to 80% of testicular mass (Setchell and Brooks, 1998). According to Bailey et al. (1998), testicular volume and weight are highly correlated. Also, Arai et al. (1998) have suggested a possible correlation between testicular volume and testicular function which was also observed in this present study.

However, the groups of rat dosed with AZECS before or after Cd showed a remarkable improvement in testis weights and testis volumes.

Marked degeneration and atrophied seminiferous tubules, interstitial edema, degenerated and vacuolated germinal epithelium, absence of late stage germ cells, degenerated spermatogenic cells and absence of sperm bundles in most tubules was observed in group of rats that received Cd. This

agrees with the findings by many investigators (Chia et al., 1994; Zhang et al., 2001).

Just like proliferating cell nuclear antigen (PCNA), an intranuclear polypeptide involved in DNA duplication and repair (Shivji et al., 1992), Ki-67 is a well-known marker of cell proliferation. It is expressed in the nuclear matrix of cells during the late G1-, S-, G2- and M phases of the cell cycle (Sasaki et al., 1988). The expression of Ki-67 and PCNA decreased in the testes of animals treated with Cd (Ferrara et al., 2006; Falana et al., 2013).

Also observed in this study was a significant tubular diameter, length, cross sectional area, width, germinal epithelia height, numerical density, perimeter, number and a significant increase in tubular lumen of the seminiferous tubule in animals treated with Cd.

Authors have reported a marked reduction of seminiferous tubular diameter, conspicuous decrease of the tubular volume density, diameter of seminal vesicle and interstitial space (Coni et al., 1992), reduction in the relative seminiferous tubule length (Selgas et al., 1997) after Cd treatment. These reports confirm the severity of cadmium-induced damage at the testicular tissue and the seminal vesicles (John et al., 1984), which could also have led to the decrease in gross anatomical parameters observed in our work.

Testicular damage caused by Cd is attributed to compete of CdCl<sub>2</sub> with Zinc in Zinc-containing enzymes and decreased activity of testis-specific enzymes (Verboost et al., 1988; Caslino et al., 1997). In accordance with the above, Neeven et al. (2007), Benoff et al. (2008), Bench et al. (1999) and Barbara et al. (2008) have also shown that exposure to Cd would interfere with male fertility, especially by its detrimental effect on testicular function (Xu et al., 1993; Chia et al., 1994). The decrease in the testicular function could also be ascribed to the effect of Cd at the hypothalamic-pituitary-gonadal axis as suggested by Lafuente et al. (2001).

It has been indicated that intracellular Cd either bound or unbound affects the regulation of mitochondrial genes such as Hsp60 which play a role in cell protection and apoptosis (Ambily et al., 2013; Cannino et al., 2009). Different modes of cell death including necrosis, apoptotic-like cell death as well as autophagy has been associated with Cd (Templeton and Liu, 2010; Ambily et al., 2013).

As was the case with the weights and for probably similar reasons, pretreatment and posttreatment with AZECS showed a remarkable improvement in the histomorphometric parameters.

The ultimate function of the testis is to maintain male fertility (Temple, 2005). The spermatozoon is particularly vulnerable to lipid peroxidation because of the molecular anatomy of its plasma membrane. Spermatogenic cells constitute one of the body tissues that are susceptible to Cd-

induced oxidative stress. Cd exert toxicity by intracellular generation of free radicals and reactive oxygen species along with intercalation with DNA and subsequent inhibition of topoisomerase (Momparker et al., 1976; Hrdina et al., 2000). This increases oxidative stress damages the sperm membranes, proteins and DNA (Kirsi and Timo, 2001; Kalender and Yel, 2005).

This could explain the significant reduction in sperm concentration, sperm motility and normal sperm morphology along with a significantly increased abnormal sperm morphology rates, as well as significantly reduced sperm progressivity as seen in groups treated with cadmium alone.

This effect may be attributed to testicular germ cell destruction and the toxic effects of these agents on the flagellum. It also has been reported that adenosine triphosphate (ATP) is an energy source for sperm motility, and its availability may be a limiting factor responsible for loss of sperm motility in Cd-treated rats (Leon et al., 2005).

Of note is also the remarkable normalization of these parameters in the groups of rats that were pre-treated and post-treated with AZECS. The result of the study in this regard is in consonance with an earlier report by Yokoi and Mayi (2004), using an ischaemic-reperfusion experimental model in rats.

Phytochemical analysis of AZECS revealed the presence in high concentration of vitamin C, vitamin E, lycopene and polyphenolic flavonone glycosides, hesperiden, neohesperiden, nariratin, limonoid and naringin. These compounds are powerful antioxidants and free radical scavengers individually and collectively (Das et al., 2002; Yeh and Yen, 2003; Ateşşahin et al., 2006; Ilbey et al., 2009).

In 2011, Shagirtha and Pari showed that Hesperetin, a citrus flavonone, protected the testes from cadmium-induced oxidative dysfunction in rats evident by significantly elevated oxidative stress markers. This is in accordance with several other results implicating oxidative stress as a mechanism pathway in cadmium-induced testicular toxicity.

Vitamin C supplementation decreased Cd-induced toxicity (Gupta et al., 2004; Serafini and Del Rio, 2004; Kamel et al., 2014). Vitamin E is said to be the most effective chain breaking lipid soluble antioxidant, it protects various fatty acids from being peroxidized via free radical scavenging mechanism (Azzi, 2007), and has been shown to protect the testes against Cd toxicity (Hoe et al., 2006).

The normalization of the parameters in the group pre-treated and post-treated with AZECS could be due to the free radical scavenging activities of the various potent antioxidant components of these extracts, as previously shown by Salah and Abdul-Hamid (2014) after Sulfasalazine toxicity. Hence, the protective and curative effect of aqueous zest

extract of *Citrus sinensis* on cadmium-induced testicular toxicity may involve the stimulation of anti-oxidative mechanisms.

As clear as the evidence from the study is, the findings may not be directly extrapolated in higher animals (including humans).

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