Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

Testicular oxidative DNA damage induced by cyclophosphamide and the impact role of pumpkin seed extract: Descriptive histopathological, ultrastructural, and immunohistochemical analysis

Eman Mohamed Samy [1]*, Muhamad Abd-Elraouf [2], Abdel-Baset I. El-Mashad[1], Shawky A. Moustafa[1], Ahmed A. Tantawy[1]

[1] *Department of Pathology, Faculty of Veterinary Medicine, Benha University, Toukh 13736, Egypt.*

[2] *Department of Pharmacognosy and Medicinal Plants, Faculty of Pharmacy, Al-Azhar University, Cairo 11884, Egypt.*

ARTICLE INFO

Keywords

Cyclophosphamide

PSE

Immunohistopathological

Testicular damage

Ultrastructure

Received 04/07/2024
Accepted 23/07/2024
Available On-Line 01/10/2024

ABSTRACT

Cyclophosphamide (CP) is an anticancer and immunosuppressive drug associated with various complications, including cytotoxicity and infertility. This study aimed to evaluate the protective effect of pumpkin seed extract (PSE) against acute testicular damage induced by CP. Twenty-four male albino rats were divided into four equal groups: Group I, control (0.5 ml/kg saline); Group II, CP-treated group (20 mg/kg); Group III, PSE group (600 mg/ kg); Group IV, CP (20 mg/kg) + PSE (600 mg/ kg) treated group. PSE was orally given to rats for 14 successive days while CP was administered intraperitoneally for 7 consecutive days from the 8th day of the study. The intraperitoneal injection of CP-induced reduction in the testicular weight, serum testosterone levels, and sperm quality (count, motility, viability, and morphology). In addition, there were severe testicular disruption, impaired spermatogenesis, and marked apoptosis of germ cells. Ultrastructural, most germ cells appeared as irregular shrunken bodies with cytoplasmic vacuolation, mitochondrial swelling, and nuclear lysis. Spermatozoa showed marked irregularity with disrupted acrosomal cap and plasma membrane. The immunohistochemistry result confirmed this damage where there was a significant decrease in PCNA-positive germ cells. Pretreatment with PSE significantly alleviated the histological testicular changes caused by CP and enhanced the spermatogenesis process evidenced by an increased number of PCNA-positive basal germ cell nuclei compared to the CP group. Furthermore, PSE improved the serum testosterone levels and sperm quality. In conclusion, PSE pretreatment effectively preserved the testicular histoarchitecture by attenuating the testicular oxidative DNA damage induced by CP..

1. INTRODUCTION

Cyclophosphamide is one of the most commonly used agents for treating various neoplastic and non-neoplastic diseases. Despite its wide therapeutic applications, its consumption has been linked to male infertility (Ghobadi et al., 2017). CP-induced reproductive dysfunctions such as impaired spermatogenesis, azoospermia, oligozoospermia, reduced hormonal synthesis, and significant histological damage in the reproductive system in both human and experimental animals (Skinner et al., 2017; Anan et al., 2018). CP is an inactive prodrug that is metabolized in the liver by cytochrome P450, forming active metabolites, phosphoramide mustard and acrolein (Sherif, 2020). Acrolein is responsible for the increased production of reactive oxygen species (ROS) and lipid peroxidation, which are possible reasons for cyclophosphamide toxicity (Kwolek-Mirek et al., 2015). Moreover, Phosphoramide-mustard alkylates DNA through alkyl groups that react with the guanine base of DNA, and abnormal cytosine-thymine pairs are created, preventing its replication and triggering cell apoptosis (Altayli et al., 2012; Kim et al., 2012).

Phytochemicals with high antioxidant activity, when given along with chemotherapeutic agents, improve the efficacy of chemotherapeutic drugs and preserve vital tissue toxicity (Goyal et al., 2019). Pumpkin (*Cucurbita pepo L.*) seeds are a rich natural source of proteins, phytosterols, and unsaturated fatty acids like linoleic, linolenic, palmitic, and stearic. Furthermore, it contains high levels of vitamins (A and E), phenolic compounds, like carotenoids, lutein, tocopherol, and chlorophyll, as well as trace elements like zinc and selenium which are considered potent antioxidant (Peiretti et al., 2017). Pumpkin seed extract (PSE) has various health benefits, including anthelmintic, antidiabetic, antihyperplastic, antitumor, antimicrobial, and cytoprotective properties (Dotto and Chacha, 2020). In addition, the gonadoprotective effect of PSE was described by Aghaei et al. (2014) and Abarikwu et al. (2022).

Therefore, this research was assigned to evaluate the potential protective effect of pumpkin seed extract (PSE) against cyclophosphamide (CP) induced testicular injury through analysis of serum testosterone levels and sperm characteristics, as well as the histopathological, immunohistochemical, and ultrastructural examination of testes.

2. MATERIAL AND METHODS

Approval Ethics

The animal care and all the experimental procedures were approved by the Ethics Committee, Faculty of Veterinary

* Correspondence to: Eman.mohamed@fvtm.bu.edu.eg

Medicine, Benha University (Ethical No. BUFVTM 06-03-23).

2.1. Animals

Adult male Albino rats weighing 250 ± 10 g were obtained from the Center of Laboratory Animals, Faculty of Veterinary Medicine, Zagazig University, Egypt. Rats were acclimated for one week before the experiment at a temperature of approximately 25± 5 °C, humidity 60 ± 5% with a 12 h light/dark cycle and provided with standard feed and water ad libitum.

2.2. Chemicals

2.2.1. Cyclophosphamide

Cyclophosphamide (Endoxan®) was obtained from Baxter Oncology GmbH Kantstrasse2 D-33790 Halle, Germany. Each vial of Endoxan 1g contains 1.069 g cyclophosphamide monohydrate as the active ingredient.

2.2.2. Preparation of pumpkin seed extract

Pumpkin seeds found inside the pods were collected manually, and dried then ethanolic pumpkin seed extract was prepared according to Xanthopoulou et al. (2009).

2.3. Experimental design

Twenty-four rats were randomly divided into four equal groups. Group I; Control group, rats received saline as a vehicle (0.5 ml/kg bwt/ IP). Group II; CP-treated group, rats intraperitoneally injected with CP (20 mg/kg bwt) (Dönmez and Yetim, 2018). Group III; PSE group, rats orally given pumpkin seed extract (600 mg/kg bwt) (Aghaei et al., 2014). Group IV; CP (20 mg/kg bwt) + PSE (600 mg/kg bwt). In this study, PSE was given to rats in III and IV groups for 14 successive days while CP was administered in II and IV groups for 7 successive days from the 8th day of the study.

2.4. Determination of body and testicular weights

The body weights of rats in all groups were recorded both at the start and end of the experimental period. Moreover, the testes weight for each rat was also recorded at the end of the study. The relative weight of the testes (organ weight/body weight × 100) was measured for each rat in all groups (Elgawish and Abdelrazek, 2014).

2.5. Testosterone analysis

Blood samples were collected from the retro-orbital plexus of each rat into gel and clot activator tube and centrifugated to separate serum (3000 rpm for 15 min). The serum testosterone hormone level was estimated by ELISA (Teilmann et al., 2014), using commercial Randox kits.

2.6. Sperm analysis

Epididymal sperm analysis including motility, viability, count, and abnormal morphology rates was done as described previously by Jalali et al. (2012).

2.7. Histopathological study

Small tissue specimens were collected from the testes of rats in all groups and immediately fixed overnight in Bouin's solution. Five µm thick paraffin sections were routinely prepared and stained with hematoxylin and eosin stain (Bancroft and Layton, 2019).

2.8. Proliferating cell nuclear antigen immunohisto-chemistry analysis

Immunohistochemistry for proliferating cells nuclear antigen (PCNA) was carried out using a monoclonal anti-PCNA antibody (clone PC10; Dako, Denmark, Glostrup, Denmark, diluted in the ratio of 1:200) as described earlier by D'Andrea et al. (2008).

PCNA-Labeling Index (PCNA-LI) for each seminiferous tubule was estimated as a percentage of immuno-labeled cells to all basal cells. For each section, the mean ± SE was calculated (Köroğlu et al., 2019).

2.9. Transmission electron microscopic (TEM) preparation

Small pieces of the testicular tissue were fixed in 2.5% glutaraldehyde buffered with PBS (0.1 M, pH 7.2) for two hours at 4ºC. Then postfixed in 1% osmium tetroxide in the same buffer for one hour at 4ºC, after that, it was dehydrated in ethanol series, embedded in Epon 812 resin (Woods and Stirling, 2019). To ensure proper orientation, semithin sections (1µm thick) were stained with 1% toluidine blue and examined by a light microscope. Ultrathin sections were contrasted with uranyl acetate and lead citrate to be examined by a Joel 4000EX electron microscope operated at 400 kV (Akishima, Tokyo, Japan) in the Electronic Microscope Lab, Faculty of Agriculture, Mansoura University.

2.10. Statistical analysis

Statistical analyses were performed using one-way ANOVA with GraphPad Prism software version 9 (La Jolla, CA, USA) followed by Tukey's multiple comparisons test. $P \leq 0.05$ was considered statistically significant. All values were expressed as means ± S.E.

3. RESULTS

3.1. PSE attenuated CP-induced body and testicular weight changes

CP-treated rats showed significant ($p < 0.05$) reduction in final body and absolute testicular weights compared with the control group. Meanwhile, rats pretreated with PSE significantly improved body weight and absolute testicular weight. However, there was no significant difference in relative testicular weight among all groups as presented in Table 1.

Table 1 Effect of PSE on CP-induced changes in body and testicular weight.

Groups	Body weight (g)		Testes weight	
	Initial	Final	Absolute (g)	Relative (%)
Control	242.5±5.53	294.70±8.51^{ab}	1.62±0.06A^{ab}	0.555±0.01
CP	249.2±5.34	210.30±8.41^{c}	1.35±0.02^{c}	0.550±0.01
PSE	247.2±6.05	299.30±12.33^{a}	1.67±0.03^{a}	0.556±0.01
CP+PSE	256.0±5.42	263.30±11.14^{b}	1.50±0.05^{b}	0.563±0.00

The values are expressed as mean ± SE. Values with different superscripts within the same column differed significantly at $P \leq 0.05$.

3.2. PSE mitigated the CP lowering serum testosterone levels

Serum testosterone level was significantly ($p < 0.0001$) decreased in the CP-treated group compared to the control group. Meanwhile, the pretreated group with PSE showed a significant ($p < 0.001$) increase in the serum testosterone level compared to the CP-treated group. However, serum testosterone levels did not reach the level of the control group (Fig. 1).

3.3. PSE improved CP-induced abnormal sperm quality

Compared to the control group, CP caused significant decreases in sperm count ($p < 0.001$), motility ($p < 0.001$), and viability ($p < 0.0001$) with significant ($p < 0.0001$) increase in sperm abnormality rates (bent middle piece, headless tail, and twisted tail). Meanwhile, the pretreatment with PSE attenuated CP-induced abnormal sperm characteristics and minimized the toxic effects of CP on the

sperm where sperm count, motility ($p < 0.01$), and viability ($p < 0.001$) were significantly increased with a marked ($p < 0.001$) decrease in sperm abnormalities (Fig. 2).

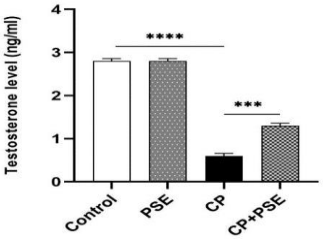

Figure (1): Effect of PSE on serum testosterone level in CP administrated rat. ****p <0.0001 vs. normal control, ***p <0.001 vs. CP group.

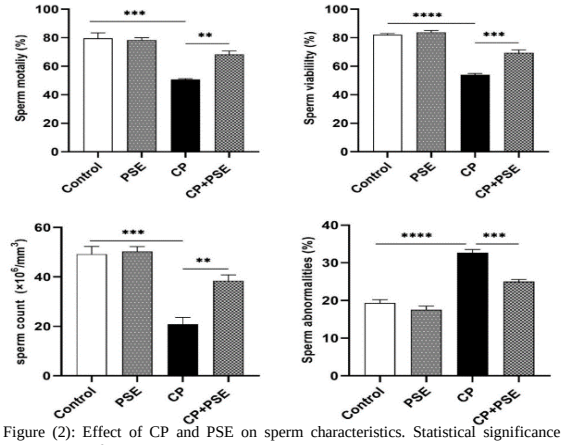

Figure (2): Effect of CP and PSE on sperm characteristics. Statistical significance is represented as follows: ****p <0.0001, ***p <0.001, **p <0.01.

3.4. PSE ameliorated the CP-induced testicular histoarchitecture damage

3.4.1. Histopathological finding

The histopathological examination of the testicular sections from the control and PSE groups showed closely packed, regular seminiferous tubules separated by narrow interstitial tissue containing Leydig cells and blood vessels. The majority of the examined testicular sections revealed that the most seminiferous tubules were lined with stratified germinal epithelial cells with abundant spermatozoa in the lumina (Fig. 3A, B). In contrast, CP-induced testicular damage where seminiferous tubules were distorted shrunken and devoid of spermatozoa. Some of these tubules were lined with apoptotic germ cells that appeared as acidophilic cells with darkly stained nuclei and separated from the irregular basement membrane (Fig. 3C). Furthermore, spermatogenesis was almost absent in most seminiferous tubules which showed intensified germ cells sloughing forming multi-nucleated giant cells (Fig. 3 D, E).

Additionally, a few seminiferous tubules showed a multinucleated formation of round spermatids with condensed marginated chromatin.

Shrunken apoptotic Leydig cells with small dark nuclei and highly eosinophilic cytoplasm were occasionally detected (Fig. 3F). Moreover, there was thickening of tunica albuginea with vascular damage including congestion of the intertubular blood vessels, thrombosis, hemorrhage, and interstitial edema (Fig. 3G). Interestingly, pretreatment with PSE significantly attenuated this testicular damage where well-organized seminiferous tubules with remarkable preservation of the germinal epithelium and active spermatogenesis were observed in most examined testicular sections. However, a few apoptotic germ cells with darkly stained nuclei and highly eosinophilic cytoplasm, along with cytoplasmic vacuolations in some germ cells were detected in some seminiferous tubules (Fig. 3H). Additionally, mild interstitial edema was occasionally observed (Fig. 3I).

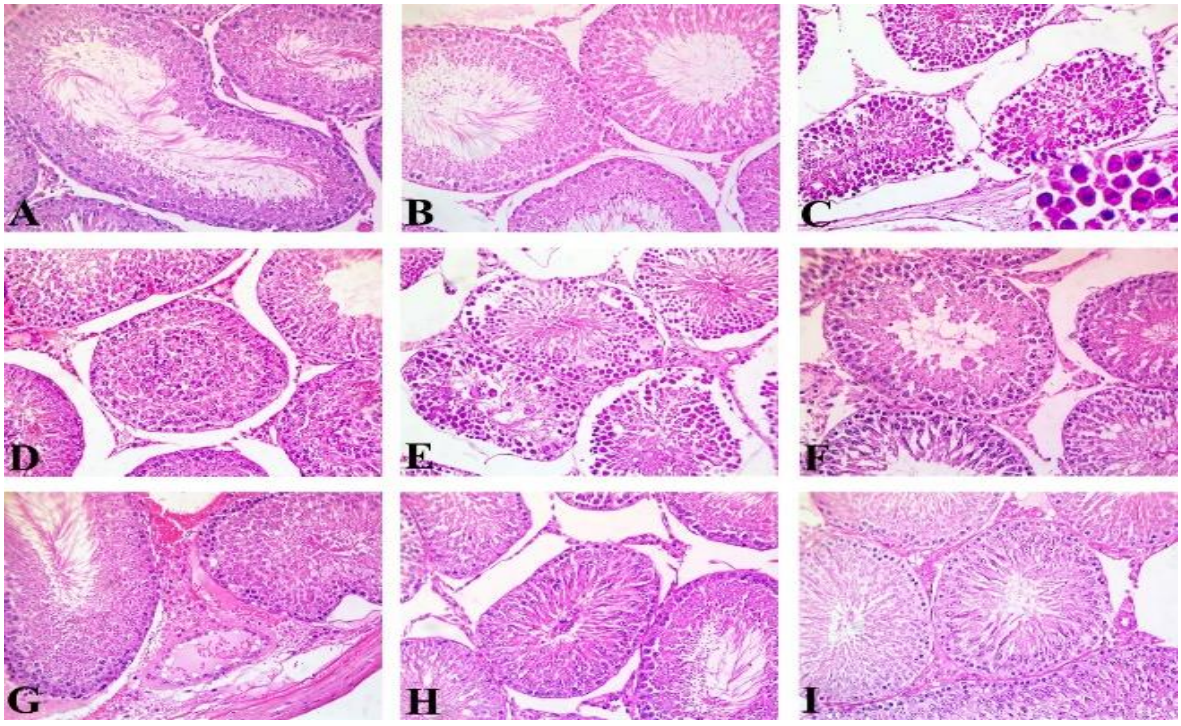

Figure (3): Representative photomicrograph of H&E-stained testicular sections of different experimental groups, x 200. (A, B): The control and PSE groups show normal histoarchitecture of testicular tissue. (C-G) CP group; (C): distorted seminiferous tubules lined with apoptotic germ cells, separated from the irregular basement membrane, (Inset: apoptotic cells showing hypereosinophilic cytoplasm with darkly stained nuclei) (D): seminiferous tubules packed with desquamated germ cells, (E): sperm giant cells formation, (F): Multinucleated formation of round spermatids and apoptotic interstitial Leydig cells. (G): recent thrombus attached to injured endothelial cells accompanied by severe perivascular hemorrhage and interstitial edema. (H, I) PSE pretreated group; (H): seminiferous tubules lined with differentiated germ cells and surrounded by regular basement membranes, few exfoliated and apoptotic germ cells along with minimal cytoplasmic vacuolations in other germ cells, (I): mild interstitial edema.

3.4.2. PSE preserved the germ cell from CP-induced DNA damage

The examined testicular sections in control and PSE groups revealed strong PCNA-positive nuclear immunoreaction of almost all basal spermatogenic cells of seminiferous tubules (Fig. 4A, B). Compared with the control group, there was decreased expression of PCNA in the basal spermatogenic cells of seminiferous tubules in the CP group (Fig. 4C). Interestingly, the most examined testicular sections of rats pretreated with PSE showed high PCNA-positive nuclear immunoreaction of basal spermatogenic cells compared with CP-treated group (Fig. 4D). The result of PCNA-LI revealed that the percentage of immunolabelled cells to all basal cells in the seminiferous tubules of the CP-treated group was significantly ($p < 0.001$) decreased compared to the control group. Meanwhile, there was a significant ($p < 0.001$) increase in PCNA-LI in the PSE pretreated group in comparison with the CP-treated group (Fig. 4E).

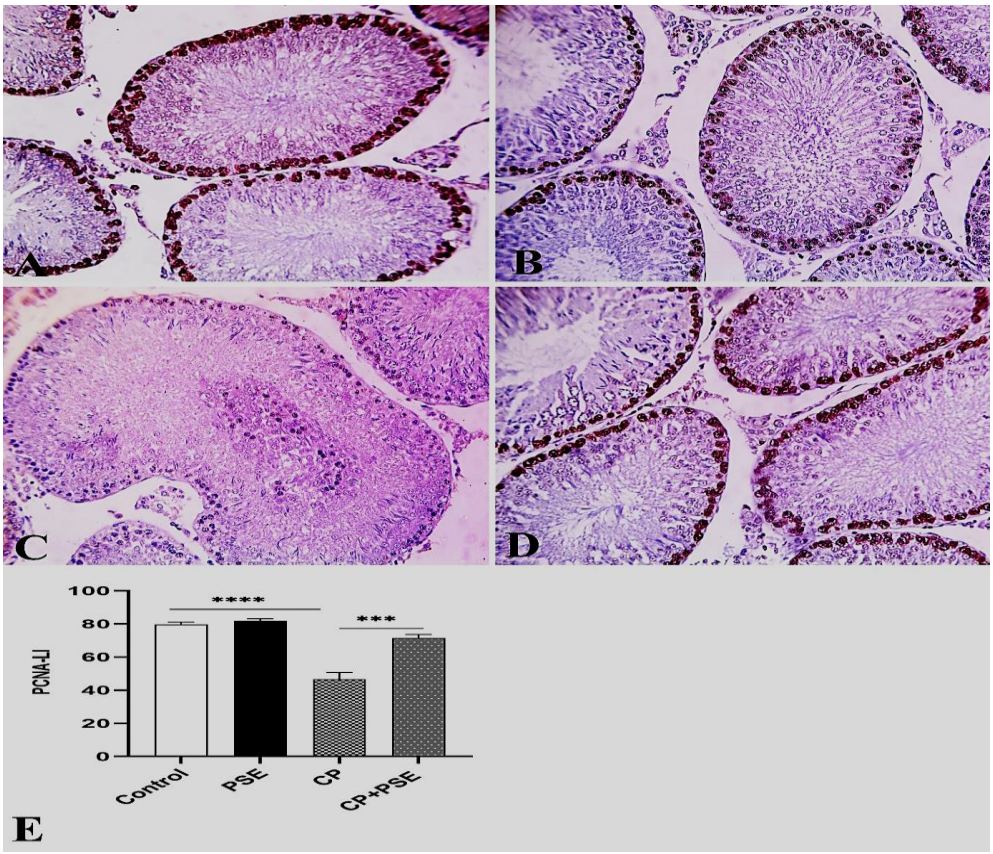

Figure (4): Representative photomicrographs of PCNA immunoexpression in the testicular tissues (x200) showing (A, B): an intensive brown nuclear positive reaction in the nuclei of almost all basal spermatogenic cells in the seminiferous tubules of rats in the control and PSE groups. (C): few numbers of spermatogonia have a positive reaction for PCNA in the testes of rats treated with CP. (D): marked PCNA immunoexpression in the nuclei of the basal spermatogenic cells of the seminiferous tubules of rats in PSE+CP. (E): PCNA-LI in different experimental groups, ****$p<0.0001$ compared to the control group and ***$p<0.001$ compared to the CP group.

3.4.3. Ultrastructural finding

Ultrastructural examination of the testicular sections of rats in control, and PSE groups displayed normal seminiferous tubules surrounded by regular basement membranes and ensheathed by a flat myoid cell. Sertoli cells appeared as pyramidal cells directly rested on the basement membrane and had an ovoid nucleus and prominent nucleolus with abundant mitochondria in their cytoplasm. The primary spermatocytes had large euchromatic nuclei with a thin rim of cytoplasm containing many small mitochondria. The spermatid cells at the Golgi phase had a rounded euchromatic nucleus covered with acrosomal vesicles. Their cytoplasm contained characteristic peripherally arranged mitochondria with centrioles and Golgi apparatus. The spermatozoa head had an electron-dense nucleus surrounded by a nuclear membrane and caudal tail sheath were intact (Fig. 5A-D).

In the CP-treated group, the seminiferous tubules displayed irregular tubular basement membranes with a large separation of germ cells from these membranes leaving a large space. Degenerated spermatogonia with cytoplasmic vacuolations were observed (Fig. 6A). Furthermore, primary spermatocytes had a pyknotic nucleus, swollen mitochondria, and vacuolated cytoplasm (Fig. 6B). Clusters of degenerated germ cells appeared as irregular shrunken bodies with cytoplasmic vacuolation, mitochondrial swelling, with absence of nucleus (Fig. 6C). The cut section of spermatozoa showed irregularity with disintegration and lysis of fibrous sheath (Fig. 6D).

In the PSE pretreatment group, most spermatogonia and spermatocytes showed intact nuclei, cytoplasmic organelles, and cell membranes. Spermatozoa mostly showed intact axoneme with only partial rupture of plasma membrane sheath. However, a few seminiferous tubules showed wrinkled and thickened basement membranes and some spermatogonia had shrunken nuclei and vacuolated mitochondria of different sizes, sometimes with ruptured cell membranes. Moreover, there were a few intercellular vacuoles between some germ cells (Fig. 7A-D).

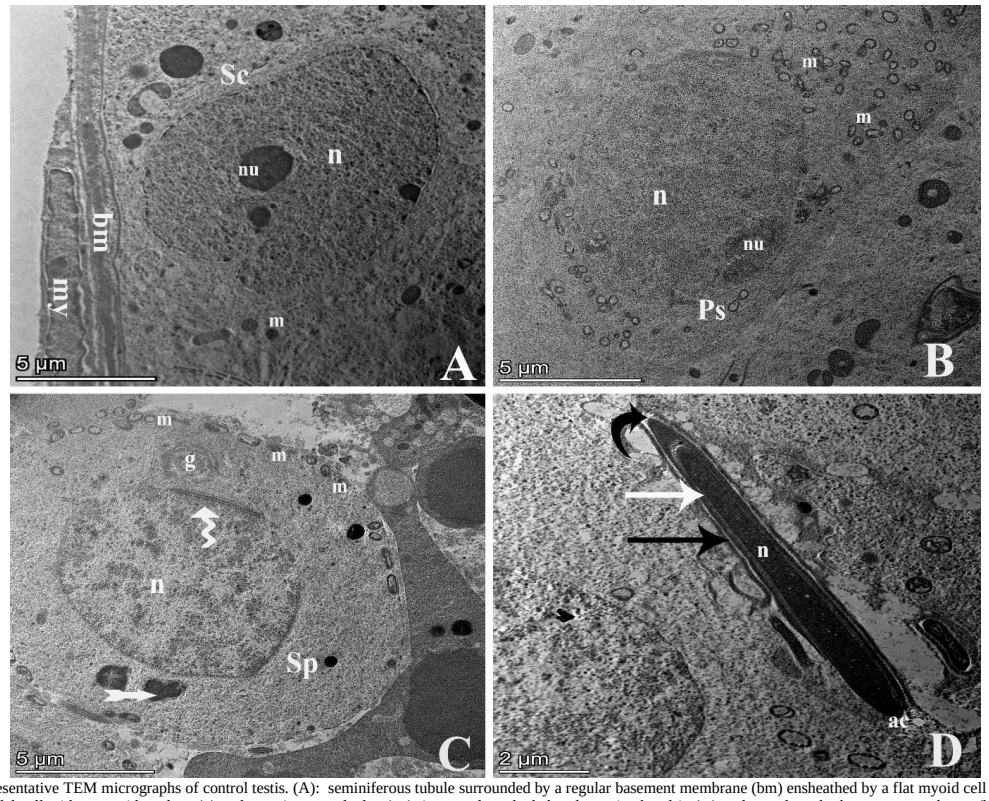

Figure (5): Representative TEM micrographs of control testis. (A): seminiferous tubule surrounded by a regular basement membrane (bm) ensheathed by a flat myoid cell (my). Sertoli cell (Sc) is a pyramidal cell with an ovoid nucleus (n) and prominent nucleolus (nu), its cytoplasm had abundant mitochondria (m) and rested on the basement membrane (bm). (B): Primary spermatocytes (PS) have large euchromatic nuclei (n) and prominent nucleolus (nu), with a thin rim of cytoplasm containing many small mitochondria (m). (C): The spermatid cell (sp) at the Golgi phase had a rounded euchromatic nucleus (n) covered with an acrosomal vesicle beginning (zigzag arrow), its nucleus surrounded with peripherally arranged mitochondria (m), its cytoplasm had centriole (tailed arrow) and Golgi apparatus (g). (D): Spermatozoal head surrounded by the intact plasma membrane (black arrow), had an electron-dense nucleus (n) surrounded by a nuclear membrane (white arrow), intact caudal tail sheath (curved arrow) and acrosomal cap (ac).

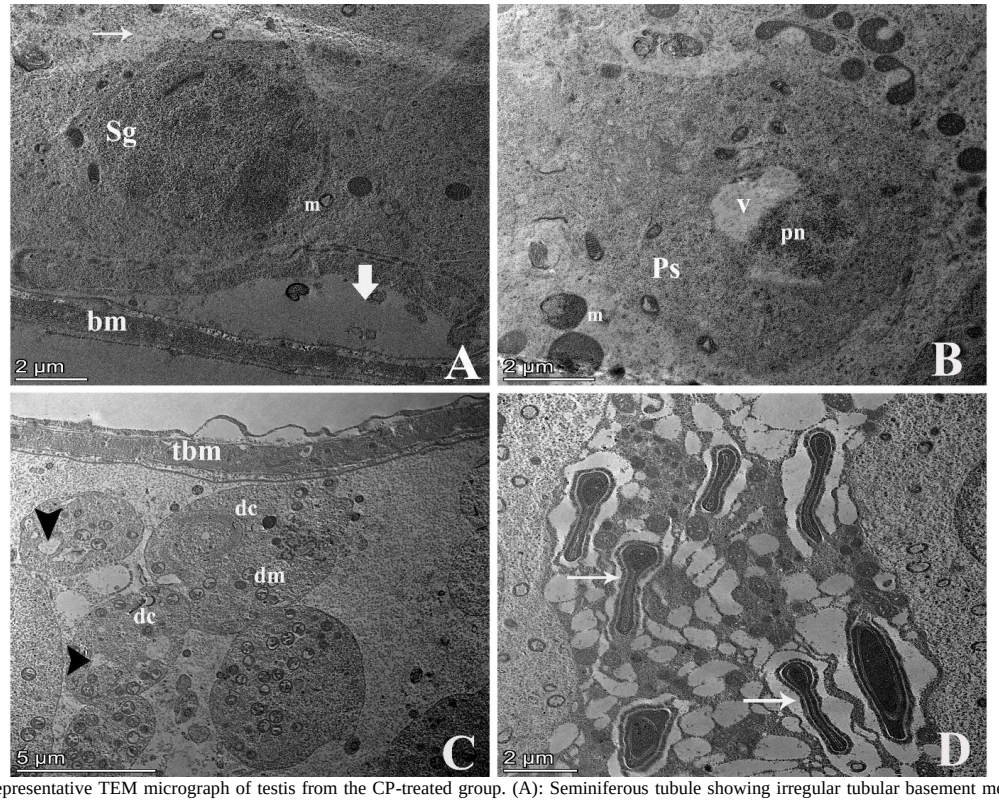

Figure (6): Representative TEM micrograph of testis from the CP-treated group. (A): Seminiferous tubule showing irregular tubular basement membrane (bm) with large separation of germ cells from basement membrane leaving large space (thick arrow), degenerated spermatogonia (Sg) with cytoplasmic vacuolations (thin arrow) and vacuolated mitochondria (m). (B): Spermatocyte (PS) with pyknotic nucleus (pn), swollen vacuolated mitochondria (m) with vacuolated cytoplasm (v). (C): Marked disintegration of seminiferous tubules with irregular, thickened basement membrane (tbm) with clusters of degenerated cells (dc). All cells appeared as irregular shrunken bodies with cytoplasmic vacuolation (arrowhead), mitochondrial swelling (dm), with absence of nucleus. (D): Spermatozoal defect with irregularity, disintegration nd lysis of fibrous sheath (white arrow).

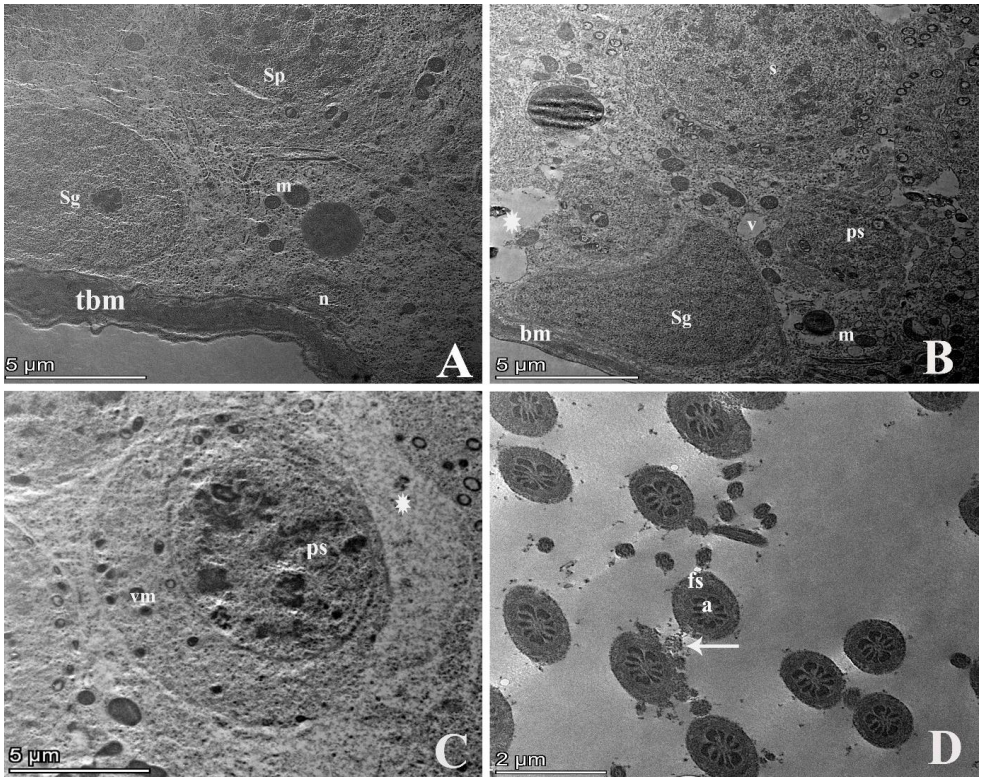

Figure (7): Representative TEM micrograph of testis from PSE pretreated group. (A): Seminiferous tubular section showing intact spermatogonia (Sg) and spermatocytes (Sp) with intact nuclei, cytoplasmic organelles and cell membrane, one necrotic spermatogonia with shrunken nucleus (n) and different sized mitochondria (m) rested on thickened, irregular basement membrane (tbm). (B): Seminiferous tubule showing intact spermatid (s), irregularly shaped spermatogonia (Sg) rested on regular basement membrane (bm), deformed spermatocytes (ps) with many cytoplasmic vacuolations (v), irregularity of mitochondria (m) and rupture of the cell membrane and increased space between cells (star). (C): Intact spermatocyte (ps) with granular cytoplasm and few vacuolated mitochondria (vm) and partial loss of tight junction with adjacent cells leaving space (star). (D): Spermatozoa mostly showing intact axoneme (a) and fibrous sheath (fs) with partial rupture of plasma membrane sheath (thin arrow).

4.DISCUSSION

Cyclophosphamide is a highly effective anticancer drug, but it is linked to various toxicities in different organs. The testes are particularly susceptible to the side effects of CP due to the presence of rapidly proliferating cells, leading to male infertility (Ghobadi et al., 2017). Numerous studies documented that natural compounds with antioxidant properties may have the ability to mitigate the testicular injury induced by cyclophosphamide (Srideepthi et al., 2024). Therefore, the present study aimed to investigate the potential effect of PSE in the mitigation of CP-induced testicular toxicity in male rats.

In the present study, the administration of CP caused decreases in body weight. These results were parallel with Afkhami-Ardakani et al. (2018). Malnutrition and decreased feed consumption after CP treatment could explain the reduction in body weight (Rezvanfar et al., 2008). Meanwhile, pre-treatment with PSE significantly inhibited the CP-induced reductions in body weight, which could be attributed to the high protein and vitamins in pumpkin seeds (Nkereuwem et al., 2011). In addition, CP-induced severe testicular damage where the most examined seminiferous tubules exhibited spermatogenesis arrest with hypocellularity, devoid of sperms, and marked apoptosis of germ cells and the interstitial Leydig cells. This result was consistent with Iqubal et al. (2020). The recorded testicular damage was reflected in the testicular weight which significantly decreased, indicating the reduction in spermatogenic cells and testosterone release (Srideepthi et al., 2024). The Leydig cell damage was reflected in serum testosterone levels which significantly decreased in the CP group, similarly reported by Alkhalaf et al. (2020). Testosterone level is essential to initiate and maintain the spermatogenesis process (Dutta et al., 2019). Therefore, the low testosterone hormone level indicates germ cell damage and impaired spermatogenesis induced by CP. The ultrastructural analysis confirmed the testicular damage induced by CP where most germ cells appeared as shrunken apoptotic bodies with nuclear lysis cytoplasmic vacuolation, and mitochondrial swelling. Furthermore, the majority of the seminiferous tubules were surrounded by thickened and irregular basement membranes. These results were consistent with Anan et al. (2018). The increased thickness of the basement membrane led to changes in its binding properties. This could impair spermatogenesis by limiting access to hormones and paracrine factors involved in spermatogenesis regulation (Aitken and Roman, 2008).

Pretreatment with PSE attenuated the testicular weight through improvement of the testicular histological integrity with normal spermatogenesis, increased differentiated spermatogenic cell mass, and abundant luminal spermatozoa in the most examined seminiferous tubules of the PSE pretreated group. These findings were consistent with Agrawal et al. (2023) who found the histological testicular alterations induced by escitalopram oxalate were improved when treated with ethanolic extract of pumpkin seed. Additionally, ultrastructurally, most germ cells of seminiferous tubules in the PSE pretreated group had intact nuclei, cytoplasmic organelles, and cell membranes. The protective effect of PSE in this study may be attributed to the antioxidant activity of PSE as it is rich in potent free radical

scavenger vitamins including A, C, and E as well as essential trace minerals such as zinc that neutralize free radical generation or directly engross the iron or copper binding sites of lipids, proteins, and DNA molecules (Shaban and Sahu, 2017). Moreover, PSE contains an abundant amount of oleic acid, a monounsaturated fatty acid, that reduces the susceptibility of the testicular tissue to lipid peroxidation (Adsul and Madkaikar, 2021). In addition, PSE enhanced spermatogenesis via improved testosterone levels which significantly increased compared to the CP-treated group. These findings were in line with Hamdi, (2020). The high testosterone levels recorded in the PSE pretreated group may be attributed to the high constituent of the unsaturated fatty acids in PSE such as omega 3, 6, and 9 that promote dehydrogenase activity which is key in testosterone production (Aghaei et al., 2014). In addition, PSE is rich in squalene and triterpene content that is related to testosterone synthesis (Nimptsch et al., 2012).

The immunohistochemical analysis of PCNA in the current study showed that the number of PCNA-positive nuclei in the basal germ cells of most examined testicular sections was significantly decreased. These results were parallel with Adana et al. (2022). This reflects the damaging effect of CP on the DNA of the germ cells as PCNA (Proliferating Cell Nuclear Antigen) plays a crucial role in DNA synthesis and cell proliferation and is commonly used as a cell proliferative marker for diagnosing germinal arrest, which results from the deterioration in DNA synthesis (Pan and Zhang, 2021). Meanwhile, the immunohistochemical analysis of PCNA- LI in the PSE pretreated group was markedly increased in the basal germinal cells compared with the CP-treated group indicating the increased proliferation rate of the germ cell with active spermatogenesis. These results were parallel with Mousa et al. (2023).

On the other hand, sperm quality including sperm count, motility, viability, and morphology are crucial indicators of spermatogenesis in the testes, sperm maturation in the epididymis, and fertility capacity assessment (Agrawal et al., 2023). In the present work, semen analysis of CP-treated rats revealed a significant reduction in sperm quality including sperm count, motility, and viability with a significant increase in morphological abnormalities (bent middle piece, headless tail, and twisted tail). Ultrastructural, sperm displayed irregularity with disintegration and lysis of fibrous sheath. Similar findings were also reported by Anan et al. (2018). The sperm abnormalities could be attributed to its high sensitivity to oxidative stress induced by CP as its membrane has a high level of polyunsaturated fatty acids with low antioxidant capacity (Asadi et al., 2017). Excessive production of ROS can damage the fluidity of the sperm plasma membrane which affects sperm motility and viability (Noh et al., 2020). In addition, the direct effect of CP on sperm DNA resulted in an increasing rate of morphological defects because cellular DNA is a primary target of CP in its anti-neoplastic and toxic activity (Ilbey et al., 2009).

PSE significantly improved sperm characteristics particularly viability with a significant reduction in sperm abnormalities. These findings were parallel with Aghaei et al. (2014). The recorded ultrastructural sperm abnormalities in the CP group were decreased as most spermatozoa had intact axoneme with minimal abnormalities on the plasma membrane sheath of some sperms. PSE improves sperm quality by containing high levels of carbohydrates, which enhance sperm motility and viability via increasing glucose metabolism, resulting in ATP synthesis (Salman et al., 2008). In addition, PSE is rich in L-carnitine which improves sperm quality and initiates sperm motility (Zhu et al., 2015).

5. CONCLUSIONS

Based on the results of the present study, it could be concluded that PSE pretreatment effectively preserved the testicular histoarchitecture by attenuating the testicular oxidative DNA damage induced by CP. Furthermore, PSE enhanced the testosterone hormone levels and sperm quality. However, Further research is needed to evaluate different protection periods and dosages against CP-induced testicular damage.

6. REFERENCES

1. Abarikwu, S.O., Mgbudom-Okah, C.J., Onuah, C.L., Ogunlaja, A., 2022. Fluted pumpkin seeds protect against busulfan-induced oxidative stress and testicular injuries in adult mice. Drug and Chemical Toxicology 45,1 , 22–32. https://doi.org/10.1080/01480545.2019.1657885
2. Adana, M.Y., Imam, A., Bello, A.A., Sunmonu, O.E., Alege, E.P., Onigbolabi, O.G., Ajao, M.S., 2022. Oral thymoquinone modulates cyclophosphamide-induced testicular toxicity in adolescent Wistar rats. Andrologia 54,4 , 14368. https://doi.org/10.1111/and.14368
3. Adsul, S. and Madkaikar, V., 2021. Pumpkin ,Cucurbita pepo seed. In: Oilseeds: Health Attributes and Food Applications, Tanwar, B., Goyal, A., ,eds. . Singapore, Springer, pp. 473–506.
4. Afkhami-Ardakani, M., Hasanzadeh, S., Shahrooz, R., Delirezh, N., Malekinejad, H., 2018. Antioxidant effects of Spirulina platensis ,Arthrospira platensis on cyclophosphamide-induced testicular injury in rats. Vet. Res. Forum 9,1 , 35-41.
5. Aghaei, S., Nikzad, H., Taghizadeh, M., Tameh, A.A., Taherian, A., Moravveji, A., 2014. Protective effect of Pumpkin seed extract on sperm characteristics, biochemical parameters and epididymal histology in adult male rats treated with Cyclophosphamide. Andrologia 46,8 , 927-935. https://doi.org/10.1111/and.12175
6. Agrawal, K., Lata, S., Bhatnagar, P., 2023. Pumpkin seed ethanolic extract protects against escitalopram-induced reproductive toxicity in male mice. Asian Pacific J. Reprod., 12,2 , 71-80. https://doi.org/10.4103/2305-0500.372377
7. Aitken, R.J., Roman, S.D., 2008. Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev 1,1 , 15-24. https://doi.org/10.4161/oxim.1.1.6843
8. Alkhalaf, M.I., Alansari, W.S., Alshubaily, F.A., Alnajeebi, A.M., Eskandrani, A.A., Tashkandi, M.A., Babteen, N.A., 2020. Chemoprotective effects of inositol hexaphosphate against cyclophosphamide-induced testicular damage in rats. Sci. Rep. 10,1 , 12599. https://doi.org/10.1038/s41598-020-68608-9
9. Altayli, E., Malkoc, E., Alp, B.F., Korkmaz, A., 2012. Prevention and treatment of cyclophosphamide and ifosfamide-induced hemorrhagic cystitis. J. Molec. Pathophysiol., 1,1 , 53-62. https://doi.org/10.5455/jmp.20120321060902
10. Anan, H.H., Zidan, R.A., Abd El-Baset, S.A., Ali, M.M., 2018. Ameliorative effect of zinc oxide nanoparticles on cyclophosphamide induced testicular injury in adult rat. Tissue and Cell 54, 80-93. https://doi.org/10.1016/j.tice.2018.08.006
11. Asadi, N., Bahmani, M., Kheradmand, A., Rafieian-Kopaei, M., 2017. The Impact of Oxidative Stress on Testicular Function and the Role of Antioxidants in Improving it: A Review. J. Clin. Diagn. Res. 11,5 , IE01-IE05. https://doi.org/10.7860/JCDR/2017/23927.9886
12. Bancroft, J.D. and Layton, C., 2019. The hematoxylin and eosin. In: Bancroft's Theory and Practice of Histological Techniques, 8th Edn, Suvarna, S.K., Layton, C., Bancroft, J.D., ,eds. . Elsevier, Philadelphia, pp. 126–138.
13. D'andrea, M., Lawrence, D., Nagele, R., Wang, C., Damiano, B., 2008. PCNA indexing as a preclinical immunohistochemical biomarker for testicular toxicity. Biotechnic & Histochemistry 83,5 , 211-220. https://doi.org/10.1080/10520290802521804

14. Dönmez, D.B. and Yetim, İ., 2018. Histological examination of the role of vitamin E in cyclophosphamide-induced testicular damage in rats. Anadolu University Journal of Science and Technology -C Life Sciences and Biotechnology 7,2 , 220-226. https://doi.org/10.18036/aubtdc.364844
15. Dotto, J.M. and Chacha, J.S., 2020. The potential of pumpkin seeds as a functional food ingredient: A review. Scientific African 10, 00575. https://doi.org/10.1016/j.sciaf.2020.e00575
16. Dutta, S., Sengupta, P., Muhamad, S., 2019. Male reproductive hormones and semen quality. Asian Pacific J. Reprod., 8,5 ,189-194. https://doi.org/10.4103/2305-0500.268132
17. Elgawish, R.A.R. and Abdelrazek, H.M.A., 2014. Effects of lead acetate on testicular function and caspase-3 expression with respect to the protective effect of cinnamon in albino rats. Toxicology Reports 1, 795–801. https://doi.org/10.1016/j.toxrep.2014.10.010
18. Ghobadi, E., Moloudizargari, M., Asghari, M.H., Abdollahi, M., 2017. The mechanisms of cyclophosphamide-induced testicular toxicity and the protective agents. Expert Opin. Drug Metab. Toxicol., 13,5 , 525-536. https://doi.org/10.1080/ 17425255.2017.1277205
19. Goyal, Y., Koul, A., Ranawat, P., 2019. Ellagic acid ameliorates cisplatin toxicity in chemically induced colon carcinogenesis. Mol. Cell Biochem., 453,1-2 , 205-215. https://doi.org/10.1007/s11010-018-3446-1
20. Hamdi, H., 2020. Testicular dysfunction induced by aluminum oxide nanoparticle administration in albino rats and the possible protective role of the pumpkin seed oil. The Journal of Basic and Applied Zoology 81, 42. https://doi.org/10.1186/s41936-020-00178-8
21. Ilbey, Y.O., Ozbek, E., Simsek, A., Otunctemur, A., Cekmen, M., Somay, A., 2009. Potential chemoprotective effect of melatonin in cyclophosphamide- and cisplatin-induced testicular damage in rats. Fertil. Steril., 92,3 , 1124-1132. https://doi.org/10.1016/j.fertnstert.2008.07.1758
22. Iqubal, A., Syed, M.A., Najmi, A.K., Ali, J., Haque, S.E., 2020. Ameliorative effect of nerolidol on cyclophosphamide-induced gonadal toxicity in Swiss Albino mice: Biochemical-, histological- and immunohistochemical-based evidences. Andrologia 52,4 , 13535. https://doi.org/10.1111/and.13535
23. Jalali, A.S., Hasanzadeh, S., Malekinejad, H., 2012. Achillea millefolium inflorescence aqueous extract ameliorates cyclophosphamide-induced toxicity in rat testis: Stereological evidences. Chinese Journal of Natural Medicines 10,4 , 247-254. https://doi.org/10.1016/S1875-5364,12 60050-8
24. Kim, W., Kim, S.H., Park, S.K., Chang, M.S., 2012. Astragalus membranaceus ameliorates reproductive toxicity induced by cyclophosphamide in male mice. Phytother. Res., 26,9 , 1418-1421. https://doi.org/10.1002/ptr.4756
25. Köroğlu, K.M., Çevik, Ö., Şener, G., Ercan, F., 2019. Apocynin alleviates cisplatin-induced testicular cytotoxicity by regulating oxidative stress and apoptosis in rats. Andrologia 51,4 , 13227. https://doi.org/10.1111/and.13227
26. Kwolek-Mirek, M., Zadrąg-Tęcza, R., Bednarska, S., Bartosz, G., 2015. Acrolein-induced oxidative stress and cell death exhibiting features of apoptosis in the yeast Saccharomyces cerevisiae deficient in SOD1. Cell Biochem. Biophysics 71, 1525-1536. https://doi.org/10.1007/s12013-014-0376-8
27. Mousa, H., Allam, A., Ali Mohamed, A. 2023. The Possible Protective Role of Crocin and Pumpkin Seed Oil against Lead-Induced Testicular Cytotoxicity in Adult Male Albino Rat. Hormonal, Histological and Immunohistochemical study. Benha Medical Journal 40, 1-16. https://doi.org/10.21608/ bmfj.2022.154319.1632
28. Nimptsch, K., Platz, E.A., Willett, W.C., Giovannucci, E., 2012. Association between plasma 25-OH vitamin D and testosterone levels in men. Clin. Endocrinol. ,Oxf ., 77,1 , 106-12. https://doi.org/10.1111/j.1365-2265.2012.04332.x
29. Nkereuwem, A., Eseyin, O., Udobre, S., Ebong, A., 2011. Evaluation of antioxidant activity and chemical analysis of the leaf of Telfairia occidentalis. Nigerian Journal of Pharmaceutical and Applied Science Research 1,1 , 21-28.
30. Noh, S., Go, A., Kim, D.B., Park, M., Jeon, H.W., Kim, B., 2020. Role of Antioxidant Natural Products in Management of Infertility: A Review of Their Medicinal Potential. Antioxidants ,Basel 9,10 , 957. https://doi.org/10.3390/antiox9100957
31. Pan, J. and Zhang, J., 2021. Research Progress of PCNA in Reproductive System Diseases. Evid. Based Complement Alternat. Med., 2391917. https://doi.org/10.1155/2021/2391917
32. Peiretti, P.G., Meineri, G., Gai, F., Longato, E., Amarowicz, R., 2017. Antioxidative activities and phenolic compounds of pumpkin ,Cucurbita pepo seeds and amaranth ,Amaranthus caudatus grain extracts. Natural Product Research 31,18 , 2178–2182. https://doi.org/10.1080/14786419.2017.1278597
33. Rezvanfar, M., Sadrkhanlou, R., Ahmadi, A., Shojaei-Sadee, H., Rezvanfar, M., Mohammadirad, A., Salehnia, A., Abdollahi, M., 2008. Protection of cyclophosphamide-induced toxicity in reproductive tract histology, sperm characteristics, and DNA damage by an herbal source; evidence for role of free-radical toxic stress. Hum. Exp. Toxicol., 27,12 , 901-10. https://doi.org/10.1177/0960327108102046
34. Salman, T.M., Olayaki, L.A., Oyeyemi, W.A., 2008. Aqueous extract of Telfairia occidentalis leaves reduces blood sugar and increases haematological and reproductive indices in male rats. African Journal of Biotechnology 7,14 , 2299-2303.
35. Shaban, A. and Sahu, R.P., 2017. Pumpkin Seed Oil: An Alternative Medicine. Int. J. Pharmacogn. Phytochem. Res., 9,2 , 11. https://doi.org/10.25258/phyto.v9i2.8066
36. Sherif, I., 2020. Uroprotective mechanisms of natural products against cyclophosphamide-induced urinary bladder toxicity: A comprehensive review. Acta. Sci. Pol. Technol. Aliment., 19,3 , 333-346. https://doi.org/10.17306/J.AFS.0832
37. Skinner, R., Mulder, R.L., Kremer, L. C., Hudson, M.M., Constine, L.S., Bardi, E., Boekhout, A., Borgmann-Staudt, A., Brown, M.C., Cohn, R., Dirksen, et al., 2017. Recommendations for gonadotoxicity surveillance in male childhood, adolescent, and young adult cancer survivors: a report from the International Late Effects of Childhood Cancer Guideline Harmonization Group in collaboration with the PanCareSurFup Consortium. Lancet. Oncol., 18,2 , e75–e90. https://doi.org/10.1016/S1470-2045,17 30026-8
38. Srideepthi, M., Devi, K.K., Ramya, B., Srinu, B., 2024. Cyclophosphamide Induced Testicular Toxicity Mechanisms and Ameliorative Agents. Ind. Vet J., 101,2 , 61-67. https://doi.org/10.62757/IVA.2024.101.2.61-67
39. Teilmann, A.C., Nygaard Madsen, A., Holst, B., Hau, J., Rozell, B., Abelson, K. S. P., 2014. Physiological and pathological impact of blood sampling by retro-bulbar sinus puncture and facial vein phlebotomy in laboratory mice. PLoS One 9,11 , 113225. https://doi.org/10.1371/journal.pone.0113225
40. Woods, A.E. and Stirling, J.W., 2019. Transmission electron microscopy. In: Bancroft's Theory and Practice of Histological Techniques, 8th Edn, Suvarna, S.K., Layton, C., Bancroft, J.D., ,eds. . Elsevier, Philadelphia, pp. 434–475.
41. Xanthopoulou, M.N., Nomikos, T., Fragopoulou, E., Antonopoulou, S., 2009. Antioxidant and lipoxygenase inhibitory activities of pumpkin seed extracts. Food Research International 42,5-6 , 641-646. https://doi.org/10.1016/j.foodres.2009.02.003
42. Zhu, B., Zheng, Y.F., Zhang, Y.Y., Cao, Y.S., Zhang, L., Li, X. G., Liu, T., Jiao, Z.Z., Wang, Q., Zhao, Z.G., 2015. Protective effect of L-carnitine in cyclophosphamide-induced germ cell apoptosis. J. Zhejiang Univ. Sci. B. 16,9 , 780-787. https://doi.org/10.1631/jzus.B1500015