Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2020.048

RESEARCH ARTICLE

Methanolic Extract of *Fraxinus xanthoxyloides* Attenuates Cisplatin-induced Reproductive Toxicity in Male Albino Rats

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ARTICLE HISTORY (20-067)

Received: February 17, 2020
Revised: May 03, 2020
Accepted: May 08, 2020
Published online: May 25, 2020

Key words:
Antioxidant
Cisplatin
Fraxinus xanthoxyloides
Testicular toxicity

ABSTRACT

Cisplatin is the most effective chemotherapeutic drug used to treat the several types of tumors. *Fraxinus xanthoxyloides* is a plant with several pharmacological and biological activities. This research was aimed to find the curative potential of *F. xanthoxyloides* extract to counter the cisplatin-induced testicular damage in albino rats. Male albino rats were used for this trial. Rats were distributed into eight equal groups. Group I: control, Group II: vehicle control; Group III: cisplatin (10 mg/kg i.p.); Group IV: cisplatin (10 mg/kg) and silymarin (100 mg/kg); Group V: cisplatin (10 mg/kg) and *F. xanthoxyloides* (200 mg/kg); Group VI: cisplatin (10 mg/kg) and *F. xanthoxyloides* (400 mg/kg); Group VII: *F. xanthoxyloides* (200 mg/kg) and Group VIII: *F. xanthoxyloides* (400 mg/kg). Rats were slaughtered on 46th day of the experiment and testes and blood samples were collected. Hormonal analysis, antioxidant enzymes and histopathological changes were assessed. Cisplatin treatment induced significant ($P<0.05$) reduction in LH, FSH and testosterone concentrations. Activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and glutathione reductase (GSR) were significantly ($P<0.05$) reduced while significant ($P<0.05$) increase in the thiobarbituric acid reactive substances (TBARS) level was observed. Cisplatin significantly ($P<0.05$) decreased the seminiferous tubules diameter, tunica albuginea height, epithelial height, spermatogonia, primary and secondary spermatocytes and spermatids while significantly ($P<0.05$) increased the interstitial spaces and tubular luminal diameter. Whereas *F. xanthoxyloides* restored all these damages and abnormalities to their normal level. It is concluded that *F. xanthoxyloides* extract have capability to ameliorate the cisplatin-induced testicular toxicity in male albino rats.

To Cite This Article: Ijaz MU, Khan MA, Yousaf S, Nasir S, Naz H, Anwar H, Younis T and Samad A, 2020. Methanolic extract of *Fraxinus xanthoxyloides* attenuates cisplatin-induced reproductive toxicity in male albino rats. Pak Vet J, 40(4): 489-493. http://dx.doi.org/10.29261/pakvetj/2020.048

INTRODUCTION

Cisplatin is one of the most effective chemotherapeutic agents, widely used for the treatment of testicular tumors. The drawback of cisplatin treatment is the induction of oxidative stress, which consequently disrupts the physiology and chemical composition of tissues (Yucel *et al*., 2019). Cisplatin changes the chromatin structure of sperm, proteins composition of sperm head, elevates abnormal sperm count and also causes histological and biochemical changes in testicular tissues (Ilbey *et al*., 2009). It has also been observed that cisplatin decreases the testosterone level, changes the LH and FSH concentrations and reduces sperm motility rate. Cisplatin triggers apoptosis and germ cell death of developing spermatozoa (Kata, 2013).

Cisplatin dependent chemotherapy, as the most effective chemotherapy for germ cell tumors, has damaging side effects on spermatogenesis and fertility, which may subsist permanently (Cherry *et al*., 2004). Mammalian spermatozoa have a complex composition of lipids with higher polyunsaturated fatty acids (PUFA)

content, sphingomyelins and plasmalogens. Consequently, its functional capability and elasticity depend on structure of the sperm membrane. However, PUFA present in spermatozoa may cause serious functional alterations because of their vulnerability for peroxidation (Sanocka and Kurpisz, 2004). Additionally, mitochondria of morphologically and functionally abnormal spermatozoa are a potential site for excessive ROS generation. Several investigations have reported cisplatin-induced oxidative damage and ROS production in reproductive organs (Reddy *et al*., 2016).

Plant based bioactive elements play eminent biological role as antioxidant, anti-inflammatory and anti-proliferative agents. It is proved that naturally occurring anti-oxidants in ethnomedicinal plants are effective in treating various types of diseases (Ngoungoure *et al*., 2019). *F. xanthoxyloides* also named as afghan ash is abundantly present in Morocco, Algeria, India, Afghanistan and northern areas of Pakistan (Sarfraz *et al*., 2017). Out of 24 existing genera of family Oleaceae, Fraxinus is a unique representative of subtribe Fraxininae and closely related with subtribe oleinae in tribe Oleeae (Wallander and Albert, 2000). The chemical analysis of *F. xanthoxyloides* has demonstrated that steroids, flavonoids, esters, phenols, lactams as well as terpenoids are characteristically present in this plant (Younis *et al.,* 2016b). In case of bone fractures and injuries in livestock, extracts of stem/twigs of this plant is utilized as ethnopharmacological practices (Mukerji and Manoharachary, 2006). The main objective of the present investigation was to determine the effects of *F. xanthoxyloides* extract on cisplatin-induced reproductive toxicity in male albino rats.

MATERIALS AND METHODS

Sampling of plant: *Fraxinus xanthoxyloides* was collected from Islamabad, Pakistan.

Preparation of extract: Leaves of *F. xanthoxyloides* were dried under shade for fourteen days and grinded into powder form. 1 kg dry weight of powder was soaked in crude methanol for 72 hours and this process was repeated twice. Filtration was carried out by using Whatman No. 1 filter and methanol was evaporated at 40°C in a rotary evaporator. Extract was preserved at 4°C for subsequent analysis.

Experimental design: Mature albino rats weighing 170-190g were used for experiment. All rats were divided into eight groups (six rats in each). Rats were provided with Tap water and food chaw and temperature was maintained (23 to 25ºC). Group I served as Control group. DMSO in olive oil was given to rats of Group II (1 ml/kg). 10 mg/kg Cisplatin was injected intraperitoneally on day first to group III. Group IV was treated with Cisplatin injection (as per group III) and oral dose of silymarin 100 mg/kg throughout the trial. Group V and VI were provided with oral dose of *F. xanthoxyloides* plant extract (200 mg/kg and 400mg/kg respectively) throughout the trial with Cisplatin injection (as per group III). Only *F. xanthoxyloides* extract (200 mg/kg and 400 mg/kg) was provided orally to group VII and VIII respectively. After completion of trial (45 days), rats were dissected, organs and blood were collected and preserved for further analysis. The experiment was approved by the Directorate of Graduate Studies, University of Agriculture, Faisalabad, Pakistan (DGS No. 6461-64). Animals were treated according to the guidelines for the use of laboratory animals.

Hormonal analysis: FSH (Catalog# BC 1029 Bio-Check Inc. USA) and LH (Catalog# BC 1031 Bio-Check Inc. USA) concentrations were measured from serum samples according to the instructions provided with kits. Enzyme Linked Immunosorbent Assay (ELISA) kit (Catalog# BC 1115 Bio-Check Inc. USA) was used to measure the concentrations of plasma testosterone.

Estimation of antioxidant enzymes: Procedure of Chance and Maehly (1955) was followed to determine the CAT and POD activity. Method of Kakkar *et al.* (1948) was followed to estimate the SOD activity. Procedure of Carlberg and Mannervik (1975) was followed in order to evaluate the GSR activity.

Lipid peroxidation: Protocol of Iqbal *et al.* (1996) was followed for the estimation of lipid peroxidation (TBARS).

Histopathological analysis of testicular tissues: The samples of testicular tissues were fixed in 10% formalin buffer and then dehydrated with rising grades of alcohol (80%, 90% and 100%) and finally shifted in cedar wood oil. Tissues were embedded in paraplast. Thin slices (3-4 µm) were made with the help of microtome, stained with hematoxylin-eosin and studied under microscope (Nikon, 187842, Japan) at 40x. Interstitial spaces, epithelial height, diameter, lumen of seminiferous tubules and tunica albuginea thickness were measured by operating image J2x software (Jensen, 2013).

Statistical analysis: The results are shown as Mean ± SE. Data were analyzed by Minitab software applying one-way analysis of variance (ANOVA) followed by Tukey's test. $P<0.05$ was considered as a level of significance.

RESULTS

Effect of *F. xanthoxyloides* on hormone concentrations: FSH, LH and plasma testosterone concentrations in cisplatin treated rats were significantly ($P<0.05$) decreased in comparison to control. In vehicle control group, hormonal concentrations remained same as in control group. These concentrations were restored close to normal in Cisplatin+Silymarin treated group and in Cisplatin+Fraxinus treated groups. Cisplatin+ Silymarin and Cisplatin+Fraxinus treated groups exhibited significantly ($P<0.05$) improved hormonal concentrations in comparison to cisplatin administered group. FSH, LH and testosterone concentrations were significantly ($P<0.05$) increased in only Fraxinus administered groups as compared with cisplatin administered group (Table 1).

Effect of *F. xanthoxyloides* on antioxidant enzymes activity: Activities of CAT, SOD, POD and GSR were significantly ($P<0.05$) decreased in cisplatin administered

group in comparison to control group. Enzymes activity in Fraxinus administered rats did not display significant difference in comparison to control and vehicle control groups. Cisplatin + Silymarin treated and Cisplatin + Fraxinus treated groups showed significant (P<0.05) increase in CAT, SOD, POD and GSR activities as compared to cisplatin administered group (Table 2).

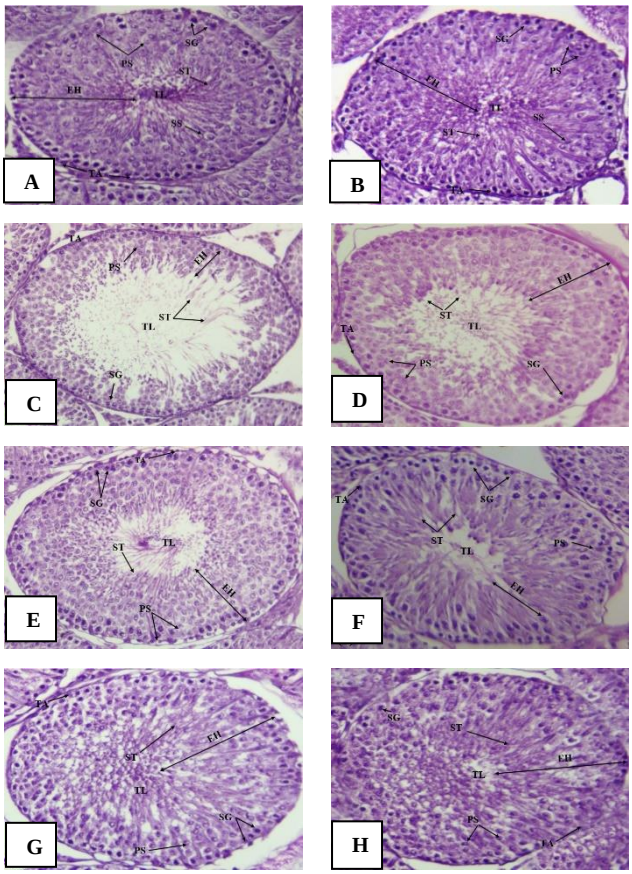

Fig. 1: Photomicrographs of testicular tissues of rats (40x; H&E): **(A)** Control; showing normal morphology of ST **(B)** Vehicle control; displaying normal morphometry. **(C)** Cisplatin administered group; displaying enlarged size of lumen **(D)** Cisplatin +Silymarin administered group: displaying recovery of injured SFT **(E)** Cisplatin+*F. xanthoxyloides* co-administered group (200 mg/kg): representing recovery of SFT **(F)** Co-administered group Cisplatin+*F. xanthoxyloides* (400 mg/kg): expressing more recovery **(G)** *F. xanthoxyloides* (200 mg/kg) administered group; displaying standard epithelium and seminiferous size **(H)** *F. xanthoxyloides* (400 mg/kg) administered group; showing normal diameter of seminiferous tubule and germ cells. Seminiferous Tubule (SFT), Tubular lumen (TL), Spermatids (ST). Secondary spermatocytes (SS), Primary spermatocytes (PS), Spermatogonia (SG), Tunica albuginea (TA).

Table 1: Mean±SEM of FSH, LH and testicular testosterone concentration (ng/ml in control and treated groups after 45 days of treatment (n=6/group)

Groups	FSH (ng/ml)	LH (ng/ml)	Testosterone (ng/ml)
Control	3.09±0.06 [a]	2.44±0.07 [a]	4.06±0.08 [a]
DMSO	3.08±0.05 [a]	2.33±0.09 [a]	3.97±0.07 [a]
Cisplatin	2.19±0.07 [b]	1.22±0.06 [b]	1.24±0.12 [b]
Cisplatin+ Silymarin	2.98±0.03 [a]	2.27±0.06 [a]	3.91±0.07 [a]
Cisplatin+ *F. xanthoxyloides* (200 mg/kg)	2.93±0.02 [a]	2.08±0.04 [a]	3.90±0.70 [a]
Cisplatin+ *F. xanthoxyloides* (400 mg/kg)	2.97±0.03 [a]	2.12±0.03 [a]	3.99±0.07 [a]
F. xanthoxyloides (200 mg/kg)	3.08±0.03 [a]	2.36±0.04 [a]	3.98±0.09 [a]
F. xanthoxyloides (400 mg/kg)	3.14±0.02 [a]	2.54±0.02 [a]	4.07±0.11 [a]

Values having different superscripts are significantly different.

Effect of *F. xanthoxyloides* on TBARS: TBARS level was significantly (P<0.05) raised in cisplatin administered group, when compared with control group and all other groups. Fraxinus treatment significantly (P<0.05) reduced the TBARS level as compared to cisplatin treated group. While only Fraxinus administration maintained the level of TBARS similar to the control group (Table 2).

Protective role of *F. xanthoxyloides* on histomorphology: Number of primary and secondary spermatocytes, spermatogonia and spermatids in seminiferous tubules were significantly (P<0.05) reduced in cisplatin administered group in comparison to other groups. But treatment of Fraxinus significantly (P<0.05) increased the number of the cells in seminiferous tubules (Table 3).

Administration of cisplatin significantly (P<0.05) increased the interstitial spaces between the tubules and tubular luminal diameter compared to control and other treated groups. While significant (P<0.05) decrease in tunica albuginea height, diameter and epithelial thickness of seminiferous tubules was noticed in cisplatin treated rats in comparison to control. No remarkable change between control and vehicle control was observed. Cisplatin + Silymarin treated group, Cisplatin + Fraxinus treated groups and only Fraxinus administered groups showed significant (P<0.05) reduction in interstitial spaces and tubular luminal diameter compared to cisplatin administered group. Cisplatin+Silymarin treated group, Cisplatin+Fraxinus treated groups and Fraxinus administered groups displayed significant (P<0.05) increase in tunica albuginea height, tubular diameter and epithelial thickness in comparison to cisplatin treated group (Table 4; Fig. 1).

DISCUSSION

Investigations on different plant extracts have proved that plant-based antioxidants have capability to counter the oxidative stress. The oxidative stress is a foremost cause of various ailments in animals (Samad *et al*., 2020). Medicinal plants and plant-based drugs have been used to treat the ailments in livestock since many years (Sher *et al*., 2016). *F. xanthoxyloides* plant has a variety of different chemical elements comprising esters, flavonoids, steroids, phenols, terpenoids as well as lactams are characteristically exiting in this plant (Younis *et al.*, 2016b).

In our findings, the group of rats which was administered with cisplatin, showed less LH, FSH and plasma testosterone level. LH promotes the synthesis of testosterone while FSH binds with Sertoli cells to activate spermatogenesis (Kata, 2013). The low level of serum testosterone may be due to the direct chemical influence of cisplatin at the level of leydig cells or by dysregulating the hormones of hypothalamic-pituitary-gonadal-axis which is attributed to the oxidative stress in the animals treated with drug (Latif *et al*., 2008). On the other hand, groups treated with Fraxinus showed improved hormonal levels dose dependently. On behalf of this research, it can be denounced that *F. xanthoxyloides* extract have ability to improve the hormonal concentration in rats. It may be due to the occurrence of bio-active compounds in the Fraxinus plant extract. However, there is further need to investigate the underlying mechanism behind this increase in hormonal level followed by *F. xanthoxyloides* treatment.

Table 2: Mean ± SEM of CAT, SOD, POD, GSR and TBARS observed calculations in control and treated groups after 45 days of treatment (n=6/group).

Groups (n=3/group)	CAT (U/mg protein)	POD (nanomole)	SOD (U/mg protein)	GSR (nm NADPH oxidized/min/mg tissue)	TBARS (nm TBARS/min/mg tissue)
Control	6.46±0.23[a]	8.26±0.20[a]	6.35±0.18[a]	4.20±0.15[a]	13.96±0.15[a]
DMSO	6.42±0.20[a]	8.19±0.15[a]	6.05±0.11[a]	4.09±0.07[a]	13.78±0.24[a]
Cisplatin	2.91±0.14[b]	3.28±0.19[b]	2.19±0.10[b]	1.67±0.09[b]	20.36±0.45[b]
Cisplatin+ Silymarin	6.40±0.21[a]	8.04±0.13[a]	6.02±0.09[a]	4.05±0.07[a]	14.41±0.13[a]
Cisplatin+ *F. xanthoxyloides* (200 mg/kg)	6.55±0.20[a]	7.90±0.13[a]	4.57±1.26[c]	3.94±0.12[a]	13.97±0.17[a]
Cisplatin+ *F. xanthoxyloides* (400 mg/kg)	6.60±0.17[a]	7.95±0.10[a]	5.94±0.07[a]	4.02±0.10[a]	13.85±0.30[a]
F. xanthoxyloides (200 mg/kg)	6.54±0.22[a]	7.85±0.27[a]	6.01±0.10[a]	4.21±0.06[a]	13.59±0.18[a]
F. xanthoxyloides (400 mg/kg)	6.83±0.15[a]	8.03±0.20[a]	6.02±0.19[a]	4.39±0.13[a]	13.60±0.22[a]

Values having different superscripts are significantly different.

Table 3: Mean ± SEM of number of different cell types in each seminiferous tubule in control and treated groups after 45 days of treatment (n=6/group).

Groups (n=3/group)	Spermatogonia	Primary spermatocytes	Secondary spermatocytes	Spermatids
Control	39.00±1.15[a]	33.33±2.02[a]	30.66±1.76[a]	41.66±0.88[a]
DMSO	36.66±3.53[a]	31.33±2.73[a]	28.33±3.48[a]	40.66±2.18[a]
Cisplatin	26.00±1.52[b]	19.66±2.33[b]	17.33±1.76[b]	23.66±0.88[b]
Cisplatin +Silymarin	34.33±0.33[a]	31.00±0.57[a]	27.66±0.66[a]	35.66±1.33[a]
Cisplatin+ *F. xanthoxyloides* (200 mg/kg)	31.66±0.33[a]	29.00±1.52[a]	24.66±0.88[a]	34.66±0.33[a]
Cisplatin+ *F. xanthoxyloides* (400 mg/kg)	34.33±0.33[a]	30.66±0.66[a]	28.33±0.33[a]	36.00±1.00[a]
F. xanthoxyloides (200 mg/kg)	41.00±1.00[a]	35.33±0.33[a]	32.33±1.76[a]	42.66±1.45[a]
F. xanthoxyloides (400 mg/kg)	44.66±0.33[a]	39.00±1.73[a]	36.33±1.45[a]	47.00±1.52[a]

Values having different superscripts are significantly different.

Table 4: Mean ± SEM interstitial space, tunica albuginea height, seminiferous tubule diameter, seminiferous tubule epithelial height and tubular lumen (µm) of testes in control and treated groups after 45 days of treatment (n=6/ group).

Groups (n=3/group)	Interstitial Spaces (µm)	Tunica albuginea height (µm)	Seminiferous tubules diameter (µm)	Seminiferous tubules epithelial height (µm)	Tubular lumen (µm)
Control	7.62±0.39[a]	22.89±0.45[a]	178.52±0.59[a]	72.79±0.57[a]	9.74±0.22[a]
DMSO	6.76±0.23[a]	21.28±0.04[a]	176.65±0.39[a]	72.13±0.54[a]	9.55±0.33[a]
Cisplatin	11.29±0.70[b]	12.31±0.76[b]	166.55±0.99[b]	20.06±0.42[b]	60.79±0.77[b]
Cisplatin+ Silymarin	9.25±0.23[a]	17.52±0.76[a]	177.67±0.16[a]	51.4±0.52[a]	17.33±0.63[a]
Cisplatin+ *F. xanthoxyloides* (200 mg/kg)	9.86±0.22[a]	15.54±0.34[a]	176.03±0.38[a]	49.36±0.99[a]	21.2±1.29[a]
Cisplatin+ *F. xanthoxyloides* (400 mg/kg)	7.84±0.07[a]	18.57±0.31[a]	178.28±0.59[a]	52.68±0.47[a]	17.03±1.32[a]
F. xanthoxyloides (200 mg/kg)	7.75±0.29[a]	26.63±0.36[a]	180.46±0.58[a]	74.54±0.31[a]	12.06±0.96[a]
F. xanthoxyloides (400 mg/kg)	7.45±0.40[a]	27.46±0.89[a]	183.12±0.34[a]	77.08±0.10[a]	12.62±0.54[a]

Values having different superscripts are significantly different.

Antioxidants have capability to decrease the damaging properties of free radicals. Exogenous antioxidants contain synthetic or natural substances having radical scavenging capabilities. According to our analysis, activities of antioxidant enzymes such as CAT, SOD, POD and GSR were decreased in cisplatin administered group. The decreased activities of CAT, POD, SOD and GSR may be attributed to excessive production of hydroxyl radicals and superoxide anion (Chirino and Pedraza-Chaverri, 2009). However, methanolic extract of *F. xanthoxyloides* restored the activities of antioxidant enzymes. Presence of bioactive compounds in plant extract activates the Nrf-2/ARE (nuclear factor), which improves the expression of antioxidant enzymes (Zhang *et al*., 2019). Our results are in line with study conducted by Younis *et al*. (2016a) who reported hepatoprotective effects of methanolic extract of *F. xanthoxyloides* leaves against CCl_4 by decreasing H_2O_2 and increasing antioxidant enzymes activity.

TBARS level was increased in the cisplatin treated group. Whereas, its level was near to control group in all the Fraxinus treated groups. TBARS (biomarkers of lipid peroxidation) is produced due to lipid peroxidation of the poly unsaturated fatty acids which is the final product of this chain reaction (Ito *et al*., 2019). Unevenness between antioxidant protection system (activity of CAT, SOD and POD levels) and free radicals (such as TBARS) is a state of oxidative stress. Excessive ROS is a key factor behind high level of TBARS which indicates lipid peroxidation and permanent cell damage (Montjean *et al*., 2010). In this investigation, Fraxinus attenuated the lipid peroxidation possibly due to improving the status of antioxidant enzymes which is evinced by low level of TBARS in testicular tissues. Our results are further supported by a previous study indicating attenuating effect of flavonoid (a bioactive phytochemical) against oxidative stress in reproductive system (Tvrda *et al.*, 2019).

Cisplatin administration increased the interstitial spaces and tubular lumen while tunica albuginea, diameter as well as epithelial height of seminiferous tubules were reduced. In the Fraxinus treated rats, interstitial spaces, tubular lumen, tunica albuginea, seminiferous tubules diameter and seminiferous tubules epithelial height were remained same as in control. In testes, treatment with cisplatin enhanced histopathological damage which is consistent with earlier investigations (Ijaz *et al*., 2020). Fraxinus treatment reversed the damaged conditions towards normal. It is contemplated that flavonoid components of extract are major reason behind the seminiferous tubules recovery towards normal as indicated by Samad *et al*. (2020). Spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids were decreased in cisplatin administered group. Previous study reported that imbalance between pro-oxidants and antioxidants can lead to excessive ROS production which might cause histological damages (Adejuwon *et al.*, 2015). Our findings revealed that *F. xanthoxyloides* promoted spermatogenesis which may be due to its androgenic properties, which is also in line with increase in testosterone production followed by *F. xanthoxyloides* plant extract treatment in our study.

Conclusions: Cisplatin provoked perverted testicular profile was returned towards the normal level due to the *F. xanthoxyloides* treatment. Our results recommend the regenerative as well as repairing capability of the antioxidants present in *F. xanthoxyloides*. It is concluded that *F. xanthoxyloides* have potential to ameliorate cisplatin induced testicular damage in rats.

Acknowledgements: This research was accomplished from the funds provided by University of Agriculture, Faisalabad, Pakistan.

Authors contribution: MUI and SY conceived and designed the experiments. MAK, HA and AS performed the experiments. SN and TY helped in statistical analysis and data interpretation. MUI and HN wrote the manuscript.

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