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Original article

Methanolic extract of *Nepeta paulsenii* as an ameliorative agent against CCl₄ induced testicular damage in male albino rats

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ABSTRACT

The issue of male sexual dysfunction arises due to numerous complications linked with sperm motility, imbalance of antioxidant enzymes and hormonal levels. Carbon tetrachloride (CCl₄) is widely used as a standard toxin to induce chemical injury and toxicity. A lot of research has been performed on many species of the genus *Nepeta* *in vitro* and *in vivo* but the plant *Nepeta paulsenii* have been rarely used in this aspect. The current research was designed to explore the impacts of methanolic extract of the *Nepeta paulsenii* plant on CCl₄ induced testicular impairment in male albino rats. Forty-eight rats were distributed equally into 8 groups; Control, Vehicle control, CCl₄ treated (1 ml/kg), CCl₄ + Silymarine treated, CCl₄ + *Nepeta paulsenii* (200 mg/kg) treated, CCl₄ + *Nepeta paulsenii* (400 mg/kg), *Nepeta paulsenii* (200 mg/kg) and *Nepeta paulsenii* (400 mg/kg) treated. Blood and testicles were obtained and analyzed. To check antioxidant enzymes, hormonal concentrations, daily sperm production (DSP), protein content and histopathology various analyses were performed. CCl₄ exposure resulted in adverse morphological changes and significant (p < 0.05) reduction in testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) concentrations, the activity of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), glutathione-disulfide reductase (GSR) and glutathione S-transferase (GST) enzymes, daily sperm production (DSP), protein content and high-density lipoproteins (HDL). CCl₄ increased the TBARS and H₂O₂ production along with the cholesterol, triglyceride and low-density lipoproteins (LDL) levels, but *Nepeta paulsenii* restored these changes back to their normal state significantly (p < 0.05). Therefore, it was concluded that *Nepeta paulsenii* extract has potential to ameliorate the CCl₄ induced testicular damage.

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Abbreviations: NP, *Nepeta paulsenii*; DSP, daily sperm production; FSH, follicle stimulating hormone; LH, luteinizing hormone; CAT, catalase; POD, peroxidase; SOD, superoxide dismutase; GSR, glutathione-disulfide reductase; GST, glutathione S-transferase.

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1. Introduction

Male sterility is a prominent clinical issue distressing 30% of people all over the world (Isidori et al., 2006). Several factors such as premature ejaculation, erectile dysfunction as well as quantity and quality of sperms are disturbing the male fecundity. These damages are prompted due to excessive free-radical production in the testicles. Many clinical disorders (such as hypertension) are mostly caused in response to reactive oxygen species (ROS) that give rise to premature ejaculation and erectile dysfunction. Male sexual physiognomies are controlled by androgens; hypogonadism incited with decline of natural antioxidant capability and

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intensification of ROS might instigate the reduced sexual desire in male adults that severely tamper the carnal erotic activities (Ojo et al., 2016).

Several investigational studies indicated that CCl₄ as an absolute hepatotoxin which also induces testicular toxicity (Khan and Ahmed, 2009; Ojo et al., 2016). Metabolization of CCl₄ in body tissues produces trichloromethyl free radicals (highly reactive) which leads to lipid peroxidation of phospholipids in plasma membrane and causes pathological anomalies due to the deposition of lipid originated oxidants. CCl₄ exposure induces oxidative stress in testicular tissues, leading to reduction in antioxidant enzyme activity. Histopathological studies of testicles divulged the detrimental pathway of CCl₄ in rats. The intoxication of CCl₄ elicits the reduction of androgen in male rats. It is essential to explore antioxidants, which defy the disproportionate production of free radicals to deal with such clinical diseases and excessive ROS (Khan and Ahmed, 2009; Sahreen et al., 2013).

Plant based antioxidants have attained significant importance due to their potential medicinal properties. Several epidemiological reports have revealed that intake of plant-based foods having antioxidants are useful for health as it reduces many toxicant-induced deteriorating effects and can effectively fight with the cancer and cardiovascular diseases (Arabshahi-Delouee and Urooj, 2007). *Nepeta* L. (Lamiaceae family) includes around 300 species, that are present in Central and Southern Asia, Central and Southern Europe and in faraway East (Jamzad et al., 2003). Plants of the genus *Nepeta*, also known as Catmints, have been used as traditional medicines and some of their components showed medicinal properties. Extracts of various *Nepeta* species are used in traditional medicines (Grognet, 1990). Seeds of some species of *Nepeta* genus are used as medicine for the treatment of fever, rheumatic pains, contusions, as well as the cutaneous eruption due to their anti-asthmatic, antiseptic, and anti-inflammatory attributes (Baser et al., 2000; Miceli et al., 2005). Several researchers (*in vitro* and *in vivo*) have been performed on different species of the genus *Nepeta* but the plant *Nepeta paulesenii* is rarely used in this aspect. The present analysis was performed on male albino rats to explore the effects of methanolic extract of *Nepeta paulesenii* plant leaves on CCl₄ induced testicular damage in male rats.

2. Materials and methods

2.1. Plant collection

Nepeta paulesenii plant was collected at flowering stage in late spring in 2018 from the area of Ganga Choti, Pir-Panjal (Bagh), Pakistan administrated Kashmir.

2.1.1. Drug and plant dose preparation

The leaves of the *Nepeta paulesenii* plant were dried in the air, at room temperature and under shadow to remove moisture completely. These leaves were ground to a very fine powder and finely-ground powder (5 kg) of the plant was immersed for 72 hrs. in 10 L crude methanol for extraction. The extraction process was replicated twice with the above-mentioned process. Whatman number 1 filter was used for the filtration process and rotary evaporator was used to evaporate the methanol at 40 °C temperature under decreased pressure to acquire the sticky material. This material was fractionated on the rising polarity basis and dried under vacuum with the help of a rotary evaporator. An extract of the plant was stored at –20 °C for the quality maintenance.

CCl₄ was purchased from Sigma-Aldrich, U.S.A. and diluted in olive oil for dose preparation (CCl₄: Olive oil; 2:8 v/v). 1 ml/kg body weight dose of CCl₄ was assigned keeping in view previous literature (Shah and Khan, 2017a,b).

2.2. Experimental layout

Male Albino rats were issued from the breeding and rearing center and kept in the Animal House, University of Agriculture, Faisalabad. Sexually mature rats (160–250 g) were kept at ambient temperature, served by tap water and normal diet (pellet) in 12 hrs. dark/light rotation in 26 ± 3 °C temperature for 25 days. Precise implementation of instructions by the National Institute of Animal Health (NIH) was ensured in the whole experimental trial. Ethical Committee of University of Agriculture, Faisalabad provided approval of this research study.

Rats were distributed into 8 groups; each group comprised of 6 rats. Rats of each group were placed in separate cages. Rats in group 1 (referred as a control group) were treated with normal pellet diet and tap water. Rats in group 2 were treated by giving dimethyl sulfoxide (DMSO) and olive oil (1:1; v/v) dose@1 ml/kg orally and group 3 was provided with CCl₄ (CCl₄: Olive oil; 2:8 v/v; 1 ml/kg). After twenty-four hours. of CCl₄ treatment, silymarin dose@50 mg/kg in DMSO was given to group 4 rats. Group 5 was nursed with *Nepeta paulesenii* dose@ 200 mg/kg and 6 was served with 400 mg/kg in DMSO after twenty-four hours. of CCl₄ treatment. The dose of *Nepeta paulesenii* extract 200 mg/kg and 400 mg/kg in DMSO was given to group 7 and 8 respectively (Sajid et al., 2016).

The rats were anesthetized and beheaded. Blood was collected in sterile falcon tubes, centrifuged and stored in the Refrigerator. Both of the testes were detached and washed with normal saline (NaCl, B.P. 0.9%). Tissues were minced, homogenized with distilled water and centrifuged (3000 rpm for about 15 min).

2.3. Hormone analysis

Samples of serum were collected from the experimental groups to determine the concentration of the testosterone hormone via enzyme-linked immuno sorbent assay (ELISA) kits. LH and FSH concentrations of each group were assessed from the serum samples with the help of Immunoassay Test Kits (GenWay Biotech Inc.).

2.4. Daily sperm production (DSP)

For the homogenization purpose, frozen testes were thawed at normal temperature and parenchyma was separated from tunica vaginalis. Then, its weighed amount was homogenized in five milliliter solution of 0.5% Triton X-100 and 0.9% NaCl for the 30 s via rotor, stator Homogenizer made by IKA-Werke, Staufen, Germany. Dilution of homogenate further carried out five times after preparation and 20 µL volume of the diluted homogeneous solution was shifted to Neubauer chamber and the count of spermatids of 19th stage was taken with the help of a light – microscope at 40X. An average of 3 readings was calculated for the estimation of spermatids count present in each sample. DSP was determined by obtaining the total number of spermatids/testis from these values and dividing them by 6.3 (total days these spermatids stay in the epithelial part of seminiferous tubule).

$$DSP = Y/6.3$$

Y is the spermatid numbers existing in the homogenate

2.5. Enzymatic antioxidant status

CAT and POD activity was assessed in accordance with the methodology of Chance and Maehly (1955). SOD activity was estimated by noticing the color intensity as proposed by Kakkar et al.

(1984). GSR activity was evaluated according to the method of Carlberg and Mannervik (1975) while activity of GST was measured by following the procedure of Habig et al. (1974).

2.6. Estimation of protein content

The total protein content of the testicular tissues was evaluated with the help of total protein kit (AMP Diagnostics, Austria). The values of total protein content were shown as mg/g of tissue.

2.7. TBARS (lipid peroxidation assay) and hydrogen peroxide (H₂O₂) estimation

Assessment of TBARS was carried out by the procedure of Kanter et al. (2003) with specific amendments. Assessment of H₂O₂ level was conducted by the methodology of Khan et al. (2015). Horseradish peroxidase enzyme facilitated by H₂O₂ was used.

2.7.1. Lipid profile

Estimation of HDL, LDL, triglycerides and total cholesterol with the help of AMP diagnostic kits (AMEDA labordiagnostik GmbH, Austria) by following all instructions mentioned by manufacturers on a chemistry analyzer.

2.8. Histopathological studies

CCl₄ induced testicular injuries were assessed through the histopathological examination. Samples of testicular tissues were fixed in fixing solution containing absolute alcohol (70%), formaldehyde (20%) and acetic acid (10%) followed by inserting in paraffin wax. 4–5 μm thin slices were made precisely, stained with eosin/hematoxylin and analyzed under the DIALUX-20EB light microscope (40x).

2.9. Statistical analysis

Entire data were represented as mean ± SEM. Analysis of data was performed by one-way ANOVA and means were compared by applying Tukey's test at the level of p < 0.05.

3. Results

3.1. Hormonal analysis

Concentrations of LH, FSH and testosterone were decreased significantly (p < 0.05) in CCl₄ administered rats in comparison with rats of vehicle control and control groups. Silymerine + CCl₄ served rats showed a significant increase in hormonal concentrations in comparison with CCl₄ treated group. Likewise, concentrations of hormones in CCl₄ + *N. paulsenii* treated rats were significantly improved compared to CCl₄ treated rats. Significant (p < 0.05) escalation in the concentration of hormones was evinced in a dose dependent manner in *N. paulsenii* treated groups in comparison with the CCl₄ treated rats (Table 1).

3.2. Biochemical analysis

Activity of antioxidant enzymes in comparison to control was significantly (p < 0.05) reduced in CCl₄ induced rats in comparison to control. Antioxidant enzyme activity in the *Nepeta paulsenii* treated groups displayed no significant difference in comparison to vehicle control and control groups. CCl₄ + Silymarin induced rats showed significant (p < 0.05) elevation in enzymatic activities when in comparison with CCl₄ induced group. Similarly,

CCl₄ + *Nepeta paulsenii* induced groups (dose dependent) exhibited considerable (p < 0.05) upsurge in antioxidant enzyme activity in comparison to CCl₄ treated group (Table 2).

3.3. Analysis of protein content, TBARS and H₂O₂

A significant (p < 0.05) decline was noticed in the protein content in CCl₄ administered rats when compared to vehicle control and the control group. While, protein content was regained in the CCl₄ + Silymarin treated group, as well as significant (p < 0.05) amendments were perceived in CCl₄ + *N. paulsenii* treated groups. However, no considerable changes in protein content were noticed in *N. paulsenii* treated rats in contrast to rats of the control group.

TBARS level was significantly (p < 0.05) escalated in toxicant treated group in comparison to vehicle control and control groups. TBARS level was significantly (p < 0.05) diminished in the *Nepeta paulsenii* treated rats when compared with toxicant group. CCl₄ + Silymarin induced rats also exhibited significant (p < 0.05) decline in TBARS level in comparison with CCl₄ induced group. In a similar manner, CCl₄ + *Nepeta paulsenii* treated groups (dose dependent) exhibited considerable (p < 0.05) decrease in TBARS level in comparison to CCl₄ administered group.

A significant increase was observed in H₂O₂ concentration in CCl₄ administered rats in comparison to vehicle control and control group. Concentration of H₂O₂ was normalized in the CCl₄ + Silymarin treated group. In the same spirit, H₂O₂ concentration was significantly (p < 0.05) declined in CCl₄ + *N. paulsenii* induced groups. There were not any remarkable variations in *N. paulsenii* treated rats in comparison to control group (Table 3).

3.4. Analysis of lipid profile

Numerous changes were observed in Lipid profile; HDL, LDL and triglycerides (mg/dl) in CCl₄ induced rats when compared to vehicle control and control groups. LDL and triglyceride levels were significantly raised in CCl₄ administered group in comparison to control and vehicle control group, whereas in co-treated groups, it overturns to normal state (i.e., similar to control group). *N. paulsenii* significantly decreased the levels of LDL and triglyceride in comparison to control group. Contrariwise, HDL level declined in CCl₄ treated groups when compared to control, but recovered to normal in co-treated groups. Doses of *N. paulsenii* improved high density lipid profile in damaged groups and *N. paulsenii* (only) administered group. (Table 4).

3.5. Analysis of histology and morphometric profile

Photomicrographs of male adult rat testes were studied under the lens of 40x magnification (Fig. 1). Tunica albuginea height displayed a significant (p < 0.05) reduction in CCl₄ induced group when rivalled with the control group. Conversely, the vehicle control and the control group did not exhibit any significant decline in albuginea height. *N. paulsenii* treatment significantly (p < 0.05) elevated tunica albuginea height compared to CCl₄ administered rats. In similar manner, dose dependent CCl₄ + *N. paulsenii* nursed rats exhibited a significant increase in tunica albuginea height in comparison to CCl₄ treated rats. A significant recovery in the tunica albuginea height of seminiferous tubules was perceived in co-treated rats when compared with CCl₄ treated group.

CCl₄ treatment led to a significant (p < 0.05) diminution in the thickness of seminiferous tubules in comparison to vehicle control and control groups. The seminiferous tubule diameter in *N. paulsenii* induced groups was significantly (p < 0.05) escalated when con-tested with CCl₄ administered rats. Likewise, significant expansion (p < 0.05) in diameter of seminiferous tubules in dose dependent

Table 1Effect of methanol extract of *Nepeta paulesnii* plant on plasma testosterone, LH, FSH concentrations and DSP in testes of rats against CCl₄ treatment.

| Groups n = 8 | Plasma testosterone concentration (ng/ml) | LH (mIU/ml) | FSH (mIU/ml) | DSP × 10 ⁶ /g |
|-----------------------------------|---|---------------------------|---------------------------|---------------------------|
| Control | 4.11 ± 0.05 ^a | 2.78 ± 0.10 ^a | 3.54 ± 0.06 ^a | 18.90 ± 0.77 ^a |
| DMSO | 4.11 ± 0.06 ^a | 2.77 ± 0.10 ^a | 3.56 ± 0.07 ^a | 18.56 ± 0.80 ^a |
| CCl ₄ | 1.14 ± 0.03 ^b | 1.23 ± 0.06 ^b | 2.18 ± 0.06 ^b | 6.946 ± 0.29 ^b |
| CCl ₄ + Silymarin | 3.99 ± 0.06 ^a | 2.77 ± 0.05 ^a | 3.47 ± 0.08 ^a | 17.24 ± 0.11 ^a |
| CCl ₄ + NP (200 mg/kg) | 3.88 ± 0.05 ^a | 2.05 ± 0.03 ^c | 2.93 ± 0.02 ^{ac} | 16.96 ± 0.05 ^c |
| CCl ₄ + NP (400 mg/kg) | 4.02 ± 0.09 ^a | 2.15 ± 0.04 ^a | 3.02 ± 0.34 ^{ac} | 17.47 ± 0.2 ^{ac} |
| NP (200 mg/kg) | 3.87 ± 0.09 ^a | 2.36 ± 0.04 ^{ac} | 2.81 ± 0.33 ^c | 18.57 ± 0.12 ^a |
| NP (400 mg/kg) | 4.05 ± 0.09 ^a | 2.48 ± 0.04 ^{ac} | 3.32 ± 0.12 ^a | 18.88 ± 0.10 ^a |

Values having different superscripts from CCl₄ treated group show significant difference with CCl₄ group while values having no superscript show no significant difference between CCl₄ and this group.

Table 2Effect of methanol extract of *Nepeta paulesnii* plant on catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), glutathione reductase (GSR) and glutathione S-transferase (GST) activities in testes of rats against CCl₄ treatment.

| Groups n = 8 | CAT (U/mg protein) | POD (nanomole) | SOD (U/mg protein) | GSR (nm NADPH oxidized/min/mg tissue) | GST (U/ml) |
|-----------------------------------|--------------------------|--------------------------|--------------------------|---------------------------------------|--------------------------|
| Control | 7.29 ± 0.23 ^a | 8.33 ± 0.23 ^a | 6.05 ± 0.09 ^a | 4.54 ± 0.17 ^a | 150 ± 3.42 ^a |
| DMSO | 7.10 ± 0.15 ^a | 8.25 ± 0.12 ^a | 6.02 ± 0.07 ^a | 4.47 ± 0.14 ^a | 146 ± 2.66 ^a |
| CCl ₄ | 3.30 ± 0.20 ^b | 3.52 ± 0.25 ^b | 2.03 ± 0.05 ^b | 1.70 ± 0.11 ^b | 72.8 ± 2.39 ^b |
| CCl ₄ + Silymarin | 7.05 ± 0.05 ^a | 7.62 ± 0.20 ^a | 6.03 ± 0.08 ^a | 4.17 ± 0.14 ^a | 137 ± 3.60 ^c |
| CCl ₄ + NP (200 mg/kg) | 6.67 ± 0.11 ^a | 8.03 ± 0.08 ^a | 5.54 ± 0.15 ^a | 4.03 ± 0.09 ^c | 132 ± 1.54 ^d |
| CCl ₄ + NP (400 mg/kg) | 7.05 ± 0.04 ^a | 8.07 ± 0.06 ^a | 6.00 ± 0.13 ^a | 4.09 ± 0.11 ^c | 135 ± 1.20 ^{cd} |
| NP (200 mg/kg) | 7.13 ± 0.12 ^a | 8.15 ± 0.09 ^a | 6.11 ± 0.07 ^a | 4.27 ± 0.07 ^{ac} | 140 ± 1.39 ^c |
| NP (400 mg/kg) | 7.32 ± 0.17 ^a | 8.32 ± 0.14 ^a | 6.15 ± 0.10 ^a | 4.31 ± 0.09 ^{ac} | 147 ± 0.89 ^a |

Values having different superscripts from CCl₄ treated group show significant difference with CCl₄ group while values having no superscript show no significant difference between CCl₄ and this group.

Table 3Effect of methanol extract of *Nepeta paulesnii* plant on protein content, TBARS and hydrogen peroxide (H₂O₂) concentrations in testes of rats against CCl₄ treatment.

| Groups n = 8 | Protein Content (mg/g) | TBARS (nm TBARS/min/mg tissue) | Hydrogen peroxide (H ₂ O ₂) |
|-----------------------------------|--------------------------|--------------------------------|--|
| Control | 4.92 ± 0.03 ^a | 14.65 ± 0.46 ^a | 4.18 ± 0.13 ^a |
| DMSO | 4.89 ± 0.01 ^a | 14.58 ± 0.45 ^a | 4.15 ± 0.14 ^a |
| CCl ₄ | 1.42 ± 0.06 ^b | 24.09 ± 0.59 ^b | 9.00 ± 0.06 ^b |
| CCl ₄ + Silymarin | 4.58 ± 0.02 ^a | 15.65 ± 0.20 ^a | 4.30 ± 0.12 ^a |
| CCl ₄ + NP (200 mg/kg) | 4.20 ± 0.01 ^a | 16.13 ± 0.16 ^c | 5.05 ± 0.09 ^c |
| CCl ₄ + NP (400 mg/kg) | 4.32 ± 0.02 ^a | 16.02 ± 0.12 ^c | 4.92 ± 0.07 ^{ac} |
| NP (200 mg/kg) | 4.52 ± 0.02 ^a | 15.94 ± 0.07 ^{ac} | 4.86 ± 0.07 ^{ac} |
| NP (400 mg/kg) | 4.62 ± 0.03 ^a | 15.85 ± 0.04 ^{ac} | 4.85 ± 0.06 ^{ac} |

Values having different superscripts from CCl₄ treated group show significant difference with CCl₄ group while values having no superscript show no significant difference between CCl₄ and this group.

CCl₄ + *Nepeta paulesnii* treated groups was noticed in comparison to CCl₄ induced rats.

CCl₄ treatment brings about a significant (p < 0.05) shrinkage in mean seminiferous germinal epithelial thickness as compared to

control. The thickness of germinal epithelium in the vehicle control group didn't show a significant change from the rats of the control group. However, in CCl₄ + Silymarin induced group, the height of the tubular germinal epithelium exhibited significant (p < 0.05) elevation as compared with CCl₄ administered group. Nevertheless, no remarkable alteration was displayed in the thickness of germinal epithelium in *Nepeta paulesnii* induced groups in comparison to control while in CCl₄ + *Nepeta paulesnii* treated groups, which were dose contingent, significant (p < 0.05) recovery was observed in tubular germinal epithelium height when compared with CCl₄ administered rats.

Tubular luminal diameter revealed a significant (p < 0.05) escalation in the CCl₄ induced rats in comparison to control. There was not any significant difference in the vehicle control group as compared with control. CCl₄ + Silymarin induction displayed a significant decrease in tubular luminal diameter in comparison to CCl₄ treated group. *Nepeta paulesnii* induced rats exhibited a non-significant difference in comparison with control, but a significant (p < 0.05) difference was detected as compared with CCl₄ considered group. However, CCl₄ + *Nepeta paulesnii* treated groups exhibited significant (p < 0.05) decline in diameter of lumen as compared with CCl₄ administered rats.

Table 4Effect of methanol extract of *Nepeta paulesnii* plant on HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein (LDL) and Triglyceride (mg/dl) in testes of rats against CCl₄ treatment.

| Groups n = 8 | HDL (mg/dl) | LDL (mg/dl) | Triglyceride (mg/dl) | Cholesterol Level |
|-----------------------------------|----------------------------|--------------------------|---------------------------|---------------------------|
| Control | 33.99 ± 2.62 ^a | 5.34 ± 0.17 ^a | 57.4 ± 1.55 ^a | 39.6 ± 0.88 ^a |
| DMSO | 33.78 ± 2.65 ^a | 5.34 ± 0.17 ^a | 58.0 ± 1.15 ^a | 44.0 ± 0.57 ^c |
| CCl ₄ | 16.47 ± 0.26 ^b | 15.3 ± 0.11 ^b | 104 ± 3.79 ^b | 67.8 ± 0.43 ^b |
| CCl ₄ + Silymarin | 30.40 ± 0.35 ^{ac} | 6.72 ± 0.13 ^c | 60.6 ± 1.76 ^{ac} | 45.2 ± 0.60 ^c |
| CCl ₄ + NP (200 mg/kg) | 28.22 ± 0.21 ^c | 7.05 ± 0.09 ^c | 64.6 ± 1.45 ^c | 50.2 ± 0.44 ^d |
| CCl ₄ + NP (400 mg/kg) | 28.88 ± 0.17 ^c | 6.93 ± 0.05 ^c | 63.3 ± 0.88 ^c | 47.8 ± 0.20 ^{cd} |
| NP (200 mg/kg) | 30.11 ± 0.06 ^d | 6.12 ± 0.06 ^a | 63.5 ± 0.28 ^c | 45.8 ± 0.45 ^c |
| NP (400 mg/kg) | 31.26 ± 0.21 ^d | 5.83 ± 0.07 ^a | 63.1 ± 0.51 ^c | 45.3 ± 0.33 ^c |

Values having different superscripts from CCl₄ treated group show significant difference with CCl₄ group while values having no superscript show no significant difference between CCl₄ and this group.

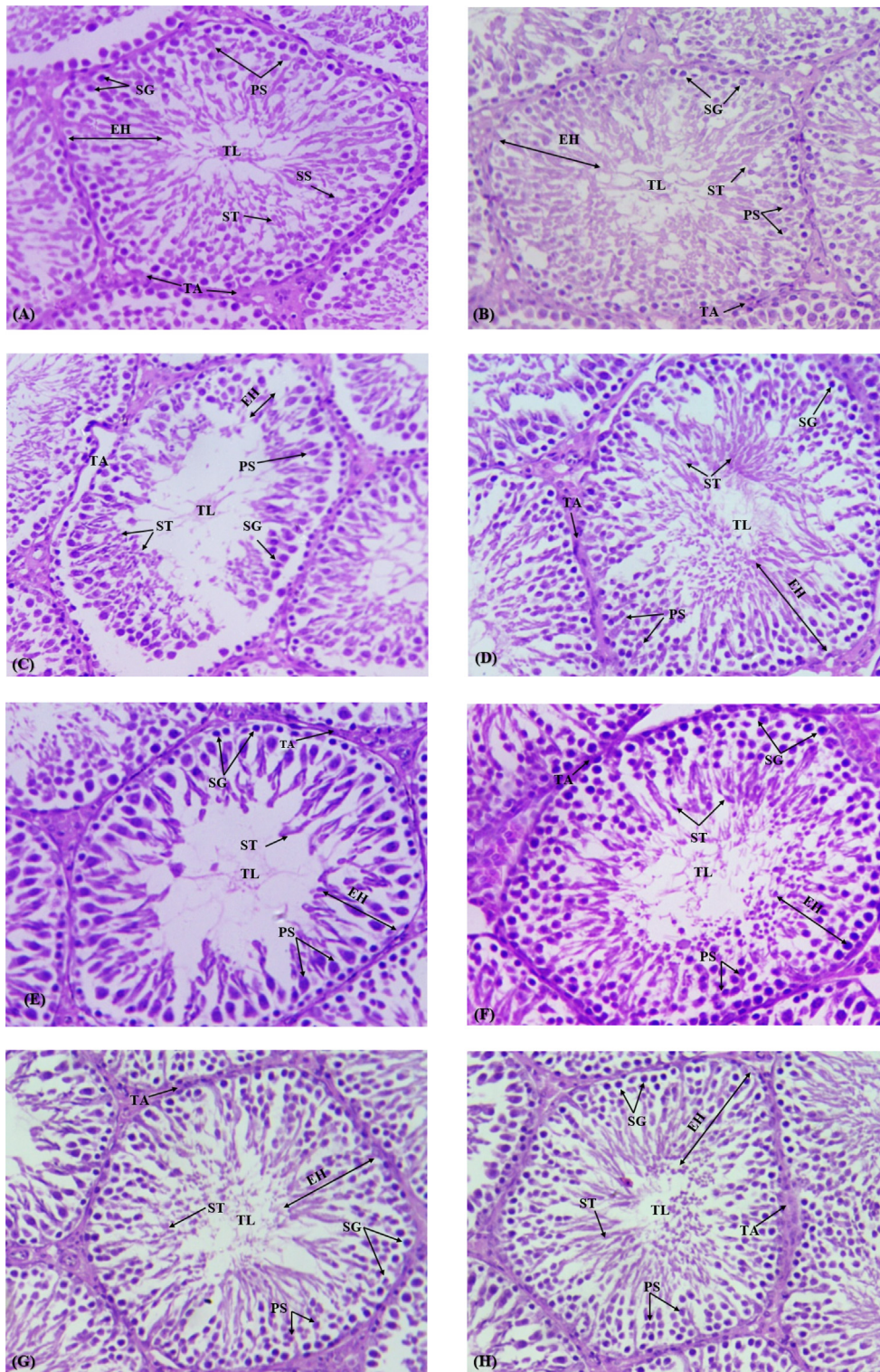


Fig. 1. Photomicrographs of male adult rat testes (H&E, 40x): (A) Control group displaying standard morphometry of seminiferous tubules with active spermatogenesis; (B) Vehicle control group also showing normal morphology of tubule. (C) CCl₄ treated group, showing increased interstitial spaces between seminiferous tubules and size of lumen (D) CCl₄ + Silymarine treated group showing restoration of damaged tubule including lumen, Tunica albuginea, diameter and epithelial height; (E) Cotreated group CCl₄ + *N. paulsenii* (200 mg/kg) presenting recovery of seminiferous tubules with germinal epithelium having proliferating germ cells at different stages such as (Spermatids, spermatocytes, spermatogonia: (F) Cotreated group CCl₄ + *N. paulsenii* (400 mg/kg) presenting more recovery of tubular lumen and germinal epithelium, increased seminiferous tubule diameter, decreased interstitial spaces: (G) *N. paulsenii* (200 mg/kg) treated groups presenting increased diameter of germinal epithelium and seminiferous comparison with control: (H) *N. paulsenii* (400 mg/kg) treated group decreased in diameter of seminiferous tubule, proliferated number of germ cells and spermatogenesis improved comparison with control group. Tunica albuginea (TA), Primary spermatocytes (PS), Spermatogonia (SG), Secondary spermatocytes (SS), Tubular lumen (TL).

Table 5
Effect of methanol extract of *Nepeta paulesenii* plant on morphometry of testes in rats against CCl₄ treatment.

| Groups n = 8 | Tunica Albuginea (μm) | Diameter of Seminiferous Tubule (μm) | Epithelial height of Seminiferous Tubule (μm) | Tubular Lumen (μm) | Interstitial Spaces (μm) |
|-----------------------------------|---------------------------|--------------------------------------|---|---------------------------|---------------------------|
| Control | 27.6 ± 0.88 ^{ac} | 177 ± 1.76 ^a | 75.6 ± 1.85 ^a | 9.66 ± 1.20 ^a | 8.66 ± 0.88 ^a |
| DMSO | 26.0 ± 0.57 ^a | 176 ± 1.45 ^a | 76.6 ± 1.20 ^a | 9.33 ± 1.20 ^a | 8.33 ± 1.45 ^a |
| CCl ₄ | 14.3 ± 1.85 ^b | 163 ± 2.40 ^b | 29.6 ± 3.48 ^b | 66.0 ± 2.64 ^b | 12.3 ± 0.88 ^b |
| CCl ₄ + Silymarin | 24.6 ± 0.88 ^a | 174 ± 1.85 ^{ac} | 56.3 ± 2.90 ^c | 23.0 ± 2.08 ^c | 9.66 ± 0.88 ^{ab} |
| CCl ₄ + NP (200 mg/kg) | 25.0 ± 1.00 ^a | 175 ± 1.73 ^{ac} | 60.0 ± 2.08 ^{cd} | 22.0 ± 1.52 ^c | 7.33 ± 0.88 ^a |
| CCl ₄ + NP (400 mg/kg) | 26.3 ± 0.66 ^a | 178 ± 2.08 ^a | 65.6 ± 0.88 ^{ad} | 17.0 ± 1.15 ^{ac} | 6.66 ± 0.66 ^{ac} |
| NP (200 mg/kg) | 28.3 ± 1.76 ^{ac} | 180 ± 1.15 ^c | 77.6 ± 1.33 ^d | 8.66 ± 1.76 ^a | 6.00 ± 0.57 ^{ac} |
| NP (400 mg/kg) | 30.3 ± 1.45 ^c | 182 ± 0.88 ^c | 78.3 ± 1.45 ^d | 10.6 ± 0.88 ^a | 5.33 ± 0.33 ^c |

Values having different superscripts from CCl₄ treated group show significant difference with CCl₄ group while values having no superscript show no significant difference between CCl₄ and this group.

A significant ($p < 0.05$) escalation was observed in the interstitial spaces between the tubules in CCl₄ administered rats in comparison to control and other groups. In vehicle control, interstitial spaces among tubules remained same as in the control. Therefore, there was not any significant difference between control and vehicle control groups. While, interstitial spaces among tubules were significantly ($p < 0.05$) decreased in the CCl₄ + Silymarin and CCl₄ + *Nepeta paulesenii* treated groups in comparison to toxicant induced rats. Interstitial spaces between the tubules displayed a significant ($p < 0.05$) reduction in *N. paulesenii* treated rats when compared with CCl₄ treated rats (Table 5).

CCl₄ induction significantly ($p < 0.05$) reduced number of spermatogonia, primary spermatocytes, secondary spermatocytes as well as spermatids in comparison to control and vehicle control groups. Whereas, doses of CCl₄ + *N. paulesenii* and Silymarin + CCl₄ significantly improved the number of these germ cells in these co-treated groups in comparison with CCl₄ treated rats. Significant ($p < 0.05$) increase in germ cell count was observed in a dose dependent manner in *N. paulesenii* medicated groups as compared with CCl₄ administered rats (Table 6).

4. Discussion

External or internal environmental disturbance due to various toxicants leads to fecundity issues in males. Control of free radicals and oxidative damages due to excess generation of ROS in the cells is a main topic of interest for scientists since last few years. The researches on natural or plant-based antioxidants have provided evidence on their health advantages in fighting against oxidative stress. This oxidative stress is among a major reason behind different disorders in living organisms (Masood et al., 2013). ROS generation stimulates hypogonadism in testicular tissues (Khan and Ahmed, 2009). CCl₄ is widely utilized as a standard toxin to induce chemical injury in various body organs for experimental purposes

(Cemek et al., 2010). Phase I cytochrome P450 converts the CCl₄ to reactive metabolic trichloromethyl radical and peroxy trichloromethyl radical through bioactivation. These free radicals make a bond with polyunsaturated fatty acids for generating alkoxy and peroxy radicals, which may lead to production of lipid peroxides. These lipid peroxides affect the activities of enzymes and result in tissue damage due to their high reactive property (Bruckner et al., 2002; Weber et al., 2003). Importance of herbal compounds proved to treat multiple disorders in animals (Kiran et al., 2018). Number of plants of the *Nepeta* genus have been used as traditional medicines and some of their components showed curative abilities (Grognet, 1990).

CCl₄, being a damaging compound, reduced the testosterone, LH and FSH concentrations and also decreased the DSP (Rajesh and Latha, 2004; Shah and Khan, 2017a,b). The low rate of sperm production in rats after CCl₄ exposure may be due to reduced level of LH, FSH and testosterone, although these hormones are essential to ensure spermatogenesis. LH instigates Leydig cells to produce testosterone and FSH incites the spermatogenesis (Horn et al., 2006; Jaffat et al., 2014). Testosterone is secreted from the interstitial cells followed by LH stimulation. The reduction in the LH level by CCl₄ exposure adversely affected the levels of plasma testosterone. CCl₄ impaired steroidogenesis and impeded the levels of testosterone. On the basis of these investigations, it can be claimed that *N. paulesenii* extract improved the hormonal concentrations and increased the DSP in rats.

CCl₄ produced damages due to free radicals by lipid peroxidation of cellular membrane, reduction of antioxidant enzymes along with their substrates, which ultimately leads to oxidative stress (Szymonik-Lesiuk et al., 2003). CCl₄ intoxication significantly reduced CAT, POD, SOD, GSR, GST and protein content, while TBARS level was significantly increased in the reproductive system of male rats. Excess quantity of H₂O₂ in the testicles due to CCl₄ attributes causes suppression of antioxidant enzymes and tissue damages (Khan and Ahmed, 2009; Sahreen et al., 2013; Ojo et al., 2016).

Table 6
Effect of methanol extract of *Nepeta paulesenii* plant on different germ cells in testes of rats against CCl₄ treatment.

| Groups n = 8 | Spermatogonia | Primary spermatocytes | Secondary spermatocytes | Spermatids |
|---|------------------------|-------------------------|-------------------------|-------------------------|
| Control | 44 ± 2.08 ^a | 41 ± 2.02 ^{ab} | 32 ± 1.76 ^a | 49 ± 1.20 ^{ab} |
| DMSO | 44 ± 1.15 ^a | 43 ± 0.88 ^{ab} | 32 ± 1.00 ^a | 48 ± 1.20 ^{ab} |
| CCl ₄ | 24 ± 1.73 ^b | 22 ± 2.08 ^c | 20 ± 1.52 ^b | 25 ± 1.85 ^c |
| CCl ₄ + Silymarin | 38 ± 1.76 ^a | 38 ± 0.88 ^b | 29 ± 1.45 ^a | 42 ± 1.52 ^b |
| CCl ₄ + <i>N. paulesenii</i> (200 mg/kg) | 44 ± 1.52 ^a | 41 ± 1.20 ^{ab} | 30 ± 0.88 ^a | 44 ± 1.73 ^{ab} |
| CCl ₄ + <i>N. paulesenii</i> (400 mg/kg) | 44 ± 2.33 ^a | 44 ± 1.45 ^{ab} | 32 ± 0.33 ^a | 45 ± 2.72 ^{ab} |
| <i>N. paulesenii</i> (200 mg/kg) | 45 ± 2.08 ^a | 48 ± 1.45 ^a | 34 ± 1.76 ^a | 49 ± 1.45 ^{ab} |
| <i>N. paulesenii</i> (400 mg/kg) | 48 ± 3.05 ^a | 50 ± 1.45 ^a | 35 ± 1.45 ^a | 51 ± 1.15 ^a |

Values having different superscripts from CCl₄ treated group show significant difference with CCl₄ group while values having no superscript show no significant difference between CCl₄ and this group.

Our other unpublished data confirm the presence of flavonoids, terpenoids, saponins and other antioxidants in the methanolic extract of *N. paulsenii* leaves. The presence of these bioactive compounds may be a key factor in reducing the free radicals. *N. paulsenii* impedes oxidation in the cells by reducing free radicals, which is revealed by low levels of H₂O₂, TBARS and escalation of total protein content along with antioxidant enzymes.

Lipid peroxidation in the testes is closely related to the dysfunction of the male reproductive system due to steroidogenesis impairment (Turner and Lysiak, 2008). Previous investigators have reported that CCl₄ exposure induced hyperlipidemia (Hsu et al., 2009; Marimuthu et al., 2013). CCl₄ intoxication significantly elevated the level of triglycerides, LDL and cholesterol, while the HDL level was reduced. Groups treated with *N. paulsenii* maintained the level of lipid profile somewhere close to control group.

CCl₄ treatment caused significant reduction in germ cells belonging to various stages while improving cell count was observed in *N. paulsenii* treated groups. Deterioration of the seminiferous tubules and germ cells was noticed in CCl₄ exposed testicles of rats. Flavonoids presence in *N. paulsenii* might be involved in recovering the CCl₄ induced damages. Similar amendments were exhibited by other medicinal plant extracts to provide shielding effect against CCl₄-instigated lesions on the testicles (Khan and Ahmed, 2009; Sahreen et al., 2013). Usage of *N. paulsenii* considerably increased germinal epithelial height of seminiferous tubules, enhanced diameter of seminiferous tubules as well as elevated tunica albuginea thickness was observed in rats treated with *N. paulsenii* extract.

5. Conclusion

The results of our investigations suggest that the *N. paulsenii* plant has curative effects against damages in testicular seminiferous tubules shape and apoptosis, histopathological lesions, hormonal disturbances as well as decreased antioxidant enzyme activity. The flavonoids terpenoids, saponins and other antioxidants present in *N. paulsenii* may have a potential to treat male sterility issues, caused by oxidative stress. Thus, it is suggested that *N. paulsenii* plant can be utilized to improve the performance of the male reproductive system and can play a major role for the betterment of humanity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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