

Therapeutic Response of *Epimedium grandiflorum*'s Different Doses to Restore the Antioxidant Potential and Reproductive Hormones in Male Albino Rats

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Abstract

Current study was planned to explore the therapeutic response of different doses of hydroethanolic extract of *Epimedium grandiflorum* leaves in male albino rats. Phytochemical analysis, HPLC and FTIR spectroscopy results revealed the presence of wide range of phenolic compounds and functional groups, respectively. Further, extract not induced significant hemolysis ($7.56 \pm 1.297\%$) against PBS ($3.65 \pm 0.35\%$) as negative control; while have significant clot lysis ($44 \pm 5.2\%$) potential, exhibited DPPH ($78.87 \pm 5.427\%$) scavenging, H_2O_2 ($31.82 \pm 3.491\%$) scavenging, antioxidant and reducing power activities. *In vivo* experimentation in albino male rats' revealed that administration of different doses (50, 100, 200 mg/Kg b.w.) of extract orally for 42 days after CCl_4 intoxication significantly ($P < 0.05$) restore the selected parameters including liver enzymes, renal profiles, and stress markers and significantly ($P < 0.05$) increased reproductive hormones like testosterone, luteinizing hormone, follicle stimulating hormone and prolactin while significantly ($P < 0.05$) decreased progesterone and estradiol toward normal in dose dependent manner. Significant ($P < 0.05$) improvement in the structural architecture of testicular tissue particularly in high dose group (200 mg/Kg b.w.) was also observed. Results revealed *E. grandiflorum* has significant therapeutic response to address the healthcare problems particularly of impotency.

Keywords

phytoconstituents, doses, therapeutic response, antioxidant potential, reproduction, diseases

Introduction

Epimedium belongs to Berberidaceae family containing about 52 species, with multiple common names like Horny Goat Weed, Rowdy Lamb Herb, Xianlinpi, Bishop's Hat, Fairy Wings, Barrenwort, and Yangheye or Yin Yang Huo.¹ *E. grandiflorum* and *Epimedium sagittatum* are the most important *Epimedium* species used to enhance sexual performance. Due to aphrodisiac properties induction in Chinese goat, *E. grandiflorum* is commonly known as horny goat weed. Icariin, a flavonol glycoside as active ingredient of this plant induce the nitric oxide (NO) synthesis in penis,² cavernosal smooth muscle where NO acts as inhibitor of cGMP-specific phosphodiesterase type 5 (PDE5),³ and boost the proliferation of smooth muscle, and reduce the formation of advanced glycation end product⁴ and caricaturist endogenous androgens.⁵ These beneficial effects of *Epimedium* species are due to the presence of various phytochemical constituents; and about over 260 including alkaloids, flavonoids, proteins, glycosides, bioactive peptides, waxes, amino acids, phyto-hormones are responsible for beneficiary activities, have been isolated. The active compounds of *Epimedium* possess a

wide range of pharmacological actions including the regulation of hormones, strengthening yang, to cure osteoporosis, boosting immunological function, hepatoprotective, anti-cancer, anti-oxidation, anti-lipidemic, anti-aging, and anti-depressant activities. Further, various *in vivo* and *in vitro* studies have been done to screen the pharmacological activities due to effective monomeric compounds or active parts present in *Epimedium*.¹

Infertility can also be referred as biological failure of an individual to contribute to conception which might be due to male or female. It is becoming one of the major problems of our

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Received 29 March 2020; received revised 21 August 2020; accepted 25 August 2020

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society and about 13-18% married couples' i.e 1 in 6 couples are affected by infertility.⁶ It was reported by WHO that infertility in worldwide affects about 60-80 million couples at reproductive age.⁷ Both in male and female dysregulation of reproductive hormones or anatomical anomalies might be the major causes of infertility.⁸ Hypothalamus-pituitary-gonadal axis and various factors like behavioral, environmental, genotoxic and genetic factors compromise the spermatogenesis in male.⁹ Phytotherapeutic approaches to treat infertility motivated to explore numerous plants and polyherbal preparations because modern drugs development is expensive and time consuming with many side effects. So in developing countries, traditional medicine plays major role in healthcare community¹⁰ and different selected parts like fruits, seeds, leaves, roots, etc or their extract powders are used to treat various diseases of humans, animals and plants.¹¹ In addition medicinal plants used as traditional medicine are easily consumable, easily available and cheaper with simple preparation strategies to mediate beneficial effects.^{12,13} Moreover, it was reported that 80% population worldwide and more than 30% of pharmaceutical formulations are dependent upon medicinal plants, reported by WHO.¹⁴ The current study was planned with the main objectives to determine the phytochemical constituents of *E. grandiflorum* leaves aqueous hydroethanolic extract, therapeutic response by determining the antioxidant parameters and reproductive hormones as well as structure of testicular tissue in CCl₄ intoxicated male albino rats.

Materials and Methods

Selection and Collection of Plant Material

E. grandiflorum leaves were purchased from local market of Faisalabad-Pakistan and were taxonomically identified and confirmed from Department of Botany, Government College University, Faisalabad-Pakistan. It was well reported that ethanol and methanol act as effective solvents for the extraction of antioxidant phenolic compounds.¹⁵ So aqueous Ethanolic (30:70) plant extract was prepared and dry leaves were sonicated twice with 10 time's higher volume of hydro alcohol for 48 hours at room temperature. Then the solution was filtered by Whatman filter paper number 1 and evaporated using rotary evaporator under reduced pressure.

Phytochemical Analysis

Qualitative analysis. Various phytochemicals including alkaloids, flavonoids, tannins, saponins, glycosides, steroids and triterpenoids were investigated in the hydroethanolic *E. grandiflorum* extract by standard methods.¹⁶⁻¹⁹

Quantitative Estimation of Phytochemicals

Total phenolic contents (TPC) and Total flavonoids contents (TFC). Folin-Ciocalteu method was used to quantify the TPC present in hydroethanolic extract of *E. grandiflorum* according to the procedure described by Jain et al., and gallic acid as standard

was used.¹⁶ The contents of total phenolics in plant extract were quantified as milligram (mg) gallic acid equivalent (GAE) per milliliter (mL) of plant hydroethanolic extract. While TFC was measured using the method explained by Pranuthi et al.¹⁷ Catechin as standard was used and TFC represented as µg CE/g (microgram Catechin per gram) of dried plants material.

High performance liquid chromatography (HPLC) analysis. HPLC was used to determine the phenolic compounds following the method with some modifications described by Xie et al.²⁰ C₁₈ column (250 × 4.6 mm internal diameter), having 5 µm film thickness, accompanying an oven set at 30°C was used in Liquid chromatography. Chromera HPLC system (Perkin Elmer, USA.) attached with Flexer Binary LC pump, UV/Vis LC Detector (Shelton CT, 06484 USA) controlled by software V. 4.2. 6410 used to analyze the data. Two solutions including solvent A consisted of acetonitrile: methanol (70:30) and solvent B composed of double distilled water with 0.5% glacial acetic acid were used as the mobile phase. UV spectra were recorded at 275 nm. Different phytochemicals were identified using 275 nm wavelength by comparing the retention times and spiking samples with standards.

Fourier-transform infrared (FTIR) spectroscopy. *E. grandiflorum* extract was milled with potassium bromide (KBr) powder and pellets were prepared by pressing. After making the pellets FTIR spectrometer (Model Bruker Platinum ATR with accessories A225/Q Platinum ATR Multiple Crystals CRY diamond and having Interferogram Size of 10550 points) in the frequency range of 400-4,000/cm was used to analyze for the identification of functional groups as representative of wide range of important phytoconstituents.

Antioxidant Profiling

Total antioxidant capacity (TAC) by phosphomolybdenum method. Phosphomolybdenum method to determine the total antioxidant capacity (TAC) of the hydroethanolic extract of *E. grandiflorum* was used, following the procedure described by Prieto et al.²¹ Different concentrations of ascorbic acid were mixed in absolute methanol to make standard solutions (1000, 500, 250, 125, 62.5 and 31.25 µg/mL). The total antioxidant activity is expressed as the number of gram equivalent of ascorbic acid.

DPPH radical scavenging potential. To evaluate the antioxidants potential of plant extract the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was used as described by Shahid et al.²² The percent DPPH inhibition was calculated using the following formula

$$\text{DPPH inhibition(\%)} = [(A_0 - A_s) / A_0] \times 100$$

Where A_s = Absorbance of Sample, A₀ = Absorbance of Blank.

Measurement of reducing power by FRAP method. Fe³⁺ (CN)₆ reduction into Fe²⁺ (CN)₆ was used to measure the reducing potential of hydroethanolic extract according to Yadav et al.²³

The absorbance at 700 nm wavelength represented the reductive potential of sample.

Hydrogen peroxide (H_2O_2) scavenging capacity. The H_2O_2 scavenging capacity of *E. grandiflorum* hydroethanolic extract was measured following the protocol described by Ruch et al.²⁴ Ascorbic acid used as standard and % age H_2O_2 scavenging activities were calculated as

$$\% \text{ Scavenged}[H_2O_2] = [(A_C - A_S) / A_C] \times 100$$

Where A_C = absorbance of the control, A_S = absorbance in the presence of the sample of extract or standards.

Hemolytic Assay to Determine Cytotoxic Potential of Plants Extract

In vitro the cytotoxic potential of hydroethanolic plant extract, 10 mg/mL was dissolved in 20% DMSO (Dimethyl sulfoxide), was determined through hemolytic assay performed by spectrophotometric method against Human erythrocytes. Triton-x-100 as positive control and phosphate buffer saline (PBS) as negative control were used.²⁵⁻²⁶ % age hemolysis was calculated as:

$$\% \text{ Hemolysis} = (A_t - A_n) / (A_c - A_n) \times 100$$

Here: A_t = Test sample absorbance, A_n = Saline control absorbance, A_c = The DMSO control absorbance.

Clot Lysis Assay to Determine Thrombolytic Activity of Plant Extract

Clot lysis method was used to evaluate the *in vitro* thrombolytic potential of hydroethanolic extract following the procedure of Rahman et al.²⁷ Streptokinase and PBS were used as positive and negative controls, respectively. The % age clot lysis activity was calculated as:

Clot lysis (%) = (clot weight before lysis – clot weight after lysis) / clot weight before lysis \times 100

In Vivo Experimentation

Animal grouping and different dosage plan. The male albino rats weighing about 160 gram to 190 gram of 8-10 weeks of age were used as animal model for *in vivo* experimentation and all the animals (n = 35) were divided into different groups with 5 animals (n = 5) in each group as follow Group 1 (GM-1): Healthy Control (Normal diet and water administered only); Group 2 (GMT-2): Intoxicated control (20% CCl_4 0.5 mL/Kg body weight (b.w) intraperitoneally (i.p.) twice a week); Group 3A (GMT-3A): Positive control (20% CCl_4 0.5 mL/ Kg b.w i.p. twice a week and Injected Testosterone enanthate 2.5 mg/Kg b.w intra muscular (i.m) twice a week); Group 3B (GMT-3B): Positive control (20% CCl_4 0.5 mL/ Kg b.w i.p. twice a week and given Laboob-e-Kabeer (Polyherbal preparation) 200mg/kg orally twice a week); Group 4A (GMT-4A): 20% CCl_4 0.5 mL/ Kg b.w i.p. twice a week and hydroethanolic plant extract (50mg/Kg b.w. as low dose) orally daily; Group

4B (GMT-4B): 20% CCl_4 0.5 mL/ Kg b.w i.p. twice a week and hydroethanolic plant extract (100mg/Kg b.w. as intermediate dose) orally daily; and Group 4C (GMT-4C): 20% CCl_4 0.5 mL/ Kg b.w i.p. twice a week and hydroethanolic plant extract (200mg/Kg b.w. as high dose) orally daily. All experimental animal groups were kept at normal diet and 12 hours light/dark cycle with normal husbandry environment for 6 weeks in the animal house, Department of Physiology, Government College University, Faisalabad-Pakistan after institutional ethical review committee.

Cytotoxic Studies

To evaluate the toxicity of hydroethanolic extract in rats' cytotoxic studies were carried out by increasing the dose from 500 mg/kg b.w. to 750 mg/kg b.w. to 1000 mg/kg b.w. It was reported that at the dose of 1000 mg/kg toxic clinical features appeared in 12 hours of drug administration. Therefore, highest dose was selected as less than one fourth (200mg/kg b.w.) of toxic level drug to run the trial.

Change in body weight and collection of blood samples. At the final day of experiment to determine the therapeutic response of plant extract, rats of each group were weighed to evaluate the change in body weight. Gain in body weight (grams) was evaluated by subtracting the pre-clinical trial body weight from post-clinical trial body weight of each group. For the determination of selected antioxidant and biochemical parameters heart puncture technique after 42 days was performed to collect blood samples. Serum from non-anti-coagulated blood was separated after centrifugation and frozen at -20°C until analysis.

Stress markers and biochemical parameters estimation. Serum stress markers including total antioxidant status (TAS), total oxidant status (TOS), Malondialdehyde (MDA) by Sahreen et al.²⁸; Superoxide dismutase (SOD) by Kakkar et al.²⁹ and Catalase (CAT) by Chance and Maehly³⁰ were determined. To evaluate the safety concerns of hydroethanolic plant extract liver enzymes like transaminases (ALT and AST) by Bergmeyer et al.,³¹ lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were determined colorimetrically as described by Thomas,³² total protein, albumin, globulin by Koller et al.,³³ and renal profile including urea by Burtis and Ashwood,³⁴ creatinine and uric acid by Swanson et al.,³⁵ were determined using automated photometric methods. The reproductive hormones including testosterone by Chen et al., (1991),³⁶ Luteinizing hormone (LH) by Frank et al., (1996),³⁷ Follicle Stimulating hormone (FSH) by Qiu et al., (1998),³⁸ Prolactin by Vanderpump et al., (1998),³⁹ progesterone by Radwanska et al., (1978)⁴⁰ and estradiol by Tsang et al., (1980)⁴¹ were measured using standard Enzyme Linked Immunosorbent Assay (ELISA) based kit method.

Histological examination of testicular tissue. Tissue samples were collected in buffered formalin (neutral) container after dissecting the animals for histological examination. After the fixation

Table 1. Qualitative Phytochemical Screening of Ethanolic Extract of *E. grandiflorum*.

Plants/phytochemicals	<i>Epimedium grandiflorum</i>
Alkaloids	+
Flavonoids	++
Tannins	+
Saponins	+
Glycosides	++
Steroids	++
Triterpenoids	+

(+) indicates the presence of phytoconstituents, (-) indicates no phytoconstituents present

Table 2. Total Phenolic, Total Flavonoid Contents, and Antioxidant Activities of Ethanolic Extract of *E. grandiflorum* (Mean \pm SEM).

Plants/phytochemicals	<i>Epimedium grandiflorum</i>
TPC (mg GAE/g dry plants material)	1010.72 \pm 12.49
TFC (μ g CE/g dry plants material)	241.8 \pm 11.18
DPPH Scavenging activity (%)	78.87 \pm 5.427
H ₂ O ₂ Scavenging activity (%)	31.82 \pm 3.491

sections were taken in labeled tissue cassettes and processed to embed in paraffin wax adopting the protocol described by Bancroft and Gamble.⁴² After embedding the tissue in paraffin wax, microtomy was performed to take thin sections and after deparaffinization, sections were stained using Hematoxylin and Eosin (H & E) stain according to the standard procedure of Bancroft and Gamble,⁴² and observed under microscope.

Statistical Analysis

The obtained results were represented as Mean \pm SEM and further interpreted through applying 1 way ANOVA test and to determine the difference between groups pairwise study was determined by Tukey's test using Minitab 17 statistical software (trial version).⁴³

Results

Phytochemical Constituents and Antioxidant Potential

Results of qualitative analysis explored different phytochemical constituents like alkaloids, flavonoids, tannins, saponins, glycosides, steroids and triterpenoids in the hydroethanolic extract of *E. grandiflorum* leaves extract (Table 1). The results of quantitative analysis revealed the total flavonoid (241.8 \pm 11.18 μ g CE/g dry plants material) and phenolic contents (1010.72 \pm 12.49 mg GAE/g dry plants material) in current study and the mean \pm SE (Standard error of mean) are given in Table 2. Free radical scavenging potentials determinations are very important parameters to evaluate the antioxidant capacity of medicinal plants. Ferric Reducing Antioxidant Power (FRAP) Assay, Phosphomolybdenum, DPPH (1,1-diphenyl-2-picrylhydrazyl) and hydrogen peroxide scavenging assays were

used to evaluate the antioxidant potential of hydroethanolic plant extract and the results represented in Table 2 and Figure 1A and B. The results revealed significant ($p < 0.05$) increase in radical scavenging and reducing activity directly correlated with the plant extracts concentrations (Figure 1A and B).

HPLC and FTIR Spectroscopy

In the present study, HPLC-UV was used to analyze the hydroethanolic extract of *E. grandiflorum* leaves qualitatively. The results revealed the presence of different phenolic compounds might be responsible for strong antioxidant potential of *E. grandiflorum* includes Gallic Acid (Rt = 2.808), Catechin (Rt = 3.257), Chlorogenic acid (Rt = 5.366), HB acid (Rt = 6.893), Caffeic acid (Rt = 7.969), Sinapic acid (Rt = 12.954) and Ferulic acid (Rt = 13.416). The results of HPLC also revealed some peaks with different retention time could not be identified; however, based on their chromatographic behaviors and UV spectra, they may correspond to unknown flavonoids compounds as presented in respective chromatogram (Figure 2).

According to infrared spectroscopy in Figure 3, there were absorption peaks at 3,500-4,000/cm, 2,918/cm, 2,360/cm, 2,341/cm, 1,726/cm, 1,586/cm, 1,494/cm, 1,462/cm, 1,426/cm, 1,428/cm, 1,296/cm, 1,259/cm, 1,179/cm, 1,120/cm, 1,023/cm, 836/cm, 726/cm, 694/cm, 563/cm and 439/cm. The absorption peaks at range 3,500 to 4,000/cm was associated with the stretching vibration of O-H, which meant that there were hydrogen bonds in intramolecules and intermolecules. The absorption peak at 2,918/cm was attributed to the asymmetrically stretching vibration of C-H. The peak at 2,360/cm and 2,341/cm might be related to stretching vibration of C-H. The absorption peak at 1,726/cm was attributed to the symmetrically stretching vibration of C=O. The absorption peaks at 1,586/cm might be related to the stretching vibration of C=N and C=C. The peaks at 1400 to 1500/cm (1,494/cm, 1,462/cm, 1,426/cm and 1,428/cm) might be attributed with strong to medium vibration of C=C and N=O functional groups. The absorption peaks at 1,296/cm and 1,259 was due to the stretching vibration of C-O. The peaks at 1,179/cm and 1,120/cm were attributed to the asymmetrically stretching vibration of C-O-C. The absorbance peak at 563/cm might be represented of aliphatic C-I stretching while the peak at 439/cm might be related to S-S stretching vibration.

Hemolytic and Thrombolytic Activities

Results of hemolytic assay revealed that the %age (percentage) hemolysis of plant extract is significantly negligible as compare to positive control (Triton-X) shown in Figure 1C. To evaluate the blood and semen thinning effect of hydroethanolic extract clot lysis activity was measured using human clotted blood. Results revealed that Streptokinase (SK; 100 μ L) incubation with clot for 90 min at 37°C dissolve the 73.6 \pm 7.7% clot while on the other hand only 3.65 \pm 0.35% clot lysis was observed in clots treated with phosphate buffer saline (PBS)

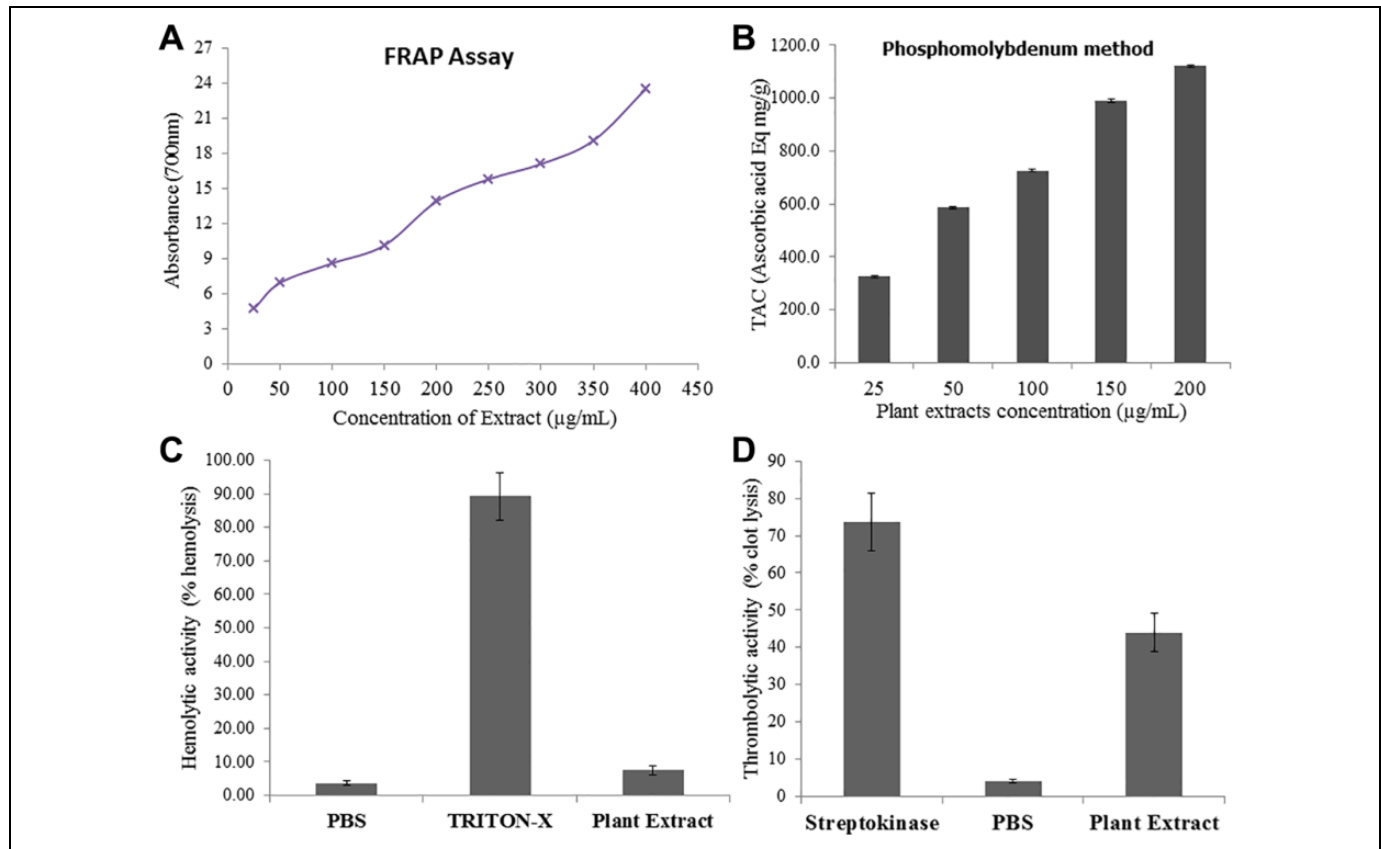


Figure 1. (A) Reducing potential of hydroethanolic extract of *E. grandiflorum* using different concentrations by FRAP method, (B) Total antioxidant capacity of hydroethanolic extract of *E. grandiflorum* by Phosphomolybdate assay (C) Thrombolytic activity of hydroethanolic extract of *E. grandiflorum* measured against streptokinase as positive control and Phosphate buffer saline (PBS) as negative control (D) Hemolytic activity of hydroethanolic extract of *E. grandiflorum* measured against Triton-X as positive control and PBS as negative control.

and significant ($p < 0.001$) thrombolytic activity of hydroethanolic extract of plant ($44 \pm 5.2\%$) was observed (Figure 1D).

In Vivo Hydroethanolic Extract' Therapeutic Response

Hepatoprotective and renoprotective activities of *E. grandiflorum* extract. Evaluation of drugs toxicity is one of the major concerns in drug development⁴⁴ because most of the herbal preparations have a wide range of health benefits so hepatoprotective and renoprotective aspects were also evaluated. The statistical analysis revealed that there was a significant ($P < 0.01$) increased in total bilirubin and liver enzymes on intoxication with CCl_4 while serum proteins significantly ($P < 0.05$) decreased. On the other hands, Significant ($P < 0.05$) improvement was reported after administration of different doses of hydroethanolic extract of plant and this improvement in liver enzymes and proteins was in dose dependent manner. Although, the concentration of globulin also increases with increased in concentration of extract amount but a significant ($P < 0.05$) difference was reported on administration of 200 mg per Kg bw extract (Figures 4A-C). The intoxication with CCl_4 administration significantly ($P < 0.05$) affects the kidney function evaluated by measuring blood urea, creatinine and uric acid level as compared to normal animal

group while administration of hydroethanolic extract of *E. grandiflorum* significantly ($P < 0.05$) restore these renal parameters on dose dependent manner (Figure 4D).

Total oxidant (TOS), antioxidant status (TAS) and stress markers in rats' blood. It was noted that induction of CCl_4 significantly ($P < 0.01$) decreased the TAS and increased the TOS in rats while the administration of plant extracts orally on daily basis revert significantly ($P < 0.01$) TAS and TOS (Table 3). The results revealed a significant ($P < 0.05$) high MDA level in CCl_4 intoxicated rats as compare to other studied animals. MDA level returned to near normal after administration of *E. grandiflorum* extract in rats (Table 3). Moreover, SOD (Superoxide dismutase) and CAT (Catalase) as enzymatic antioxidant and decreased in both enzymes activities was also noted on intoxication with CCl_4 while in plants extract and positive drugs treated rats, both SOD and CAT significantly ($P < 0.05$) improves in dose dependent manner (Table 3).

Change in body, testis weight and reproductive hormones. The changes in the results, as mean \pm SEM, of body weight (g), testis weight (g), relative gain in testis (g) and reproductive hormones in male rats have been given in Table 4 and Figure 5. The results of statistical test revealed that CCl_4 exposure

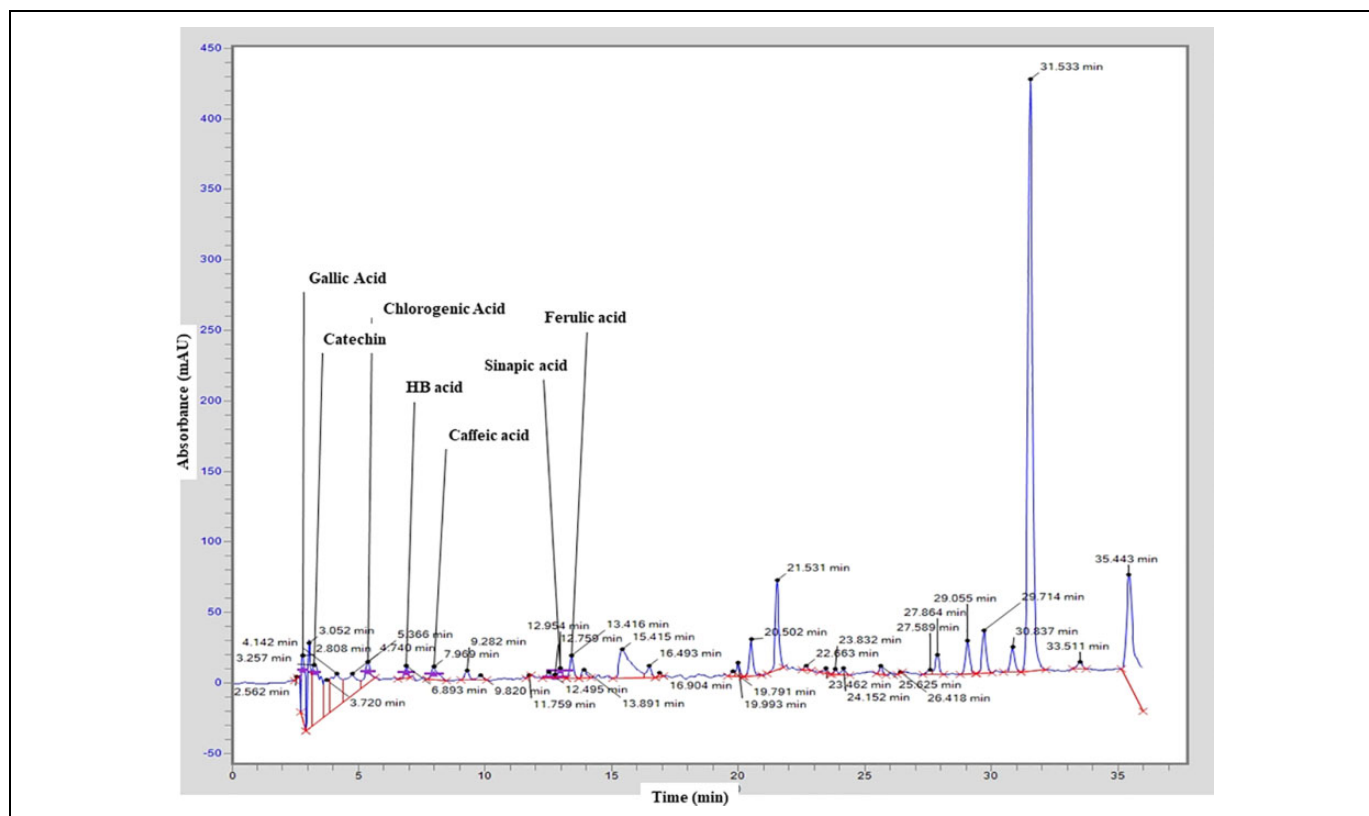


Figure 2. Chromatogram of hydroethanolic extract of *E. grandiflorum* obtained from HPLC analysis representing the presence of different phenolic molecules.

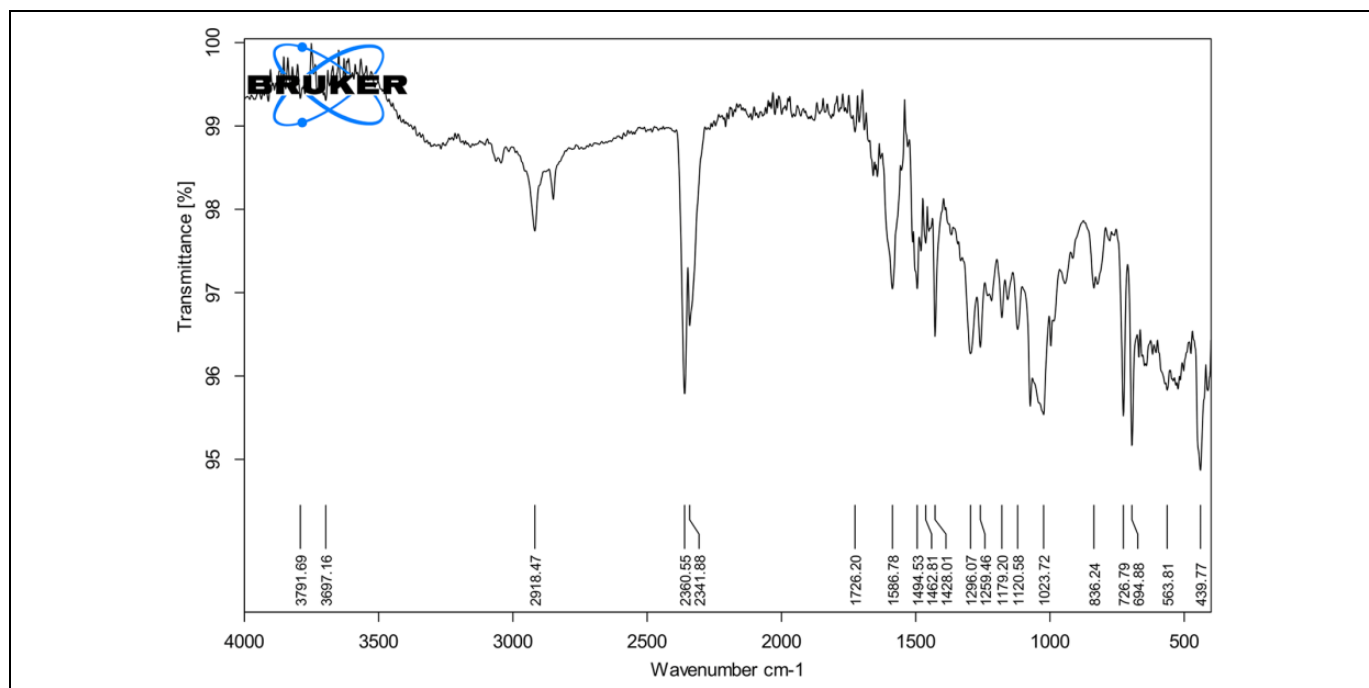


Figure 3. Infrared spectra of the hydroethanolic extract of *E. grandiflorum* leaves revealing the presence of wide range of functional groups.

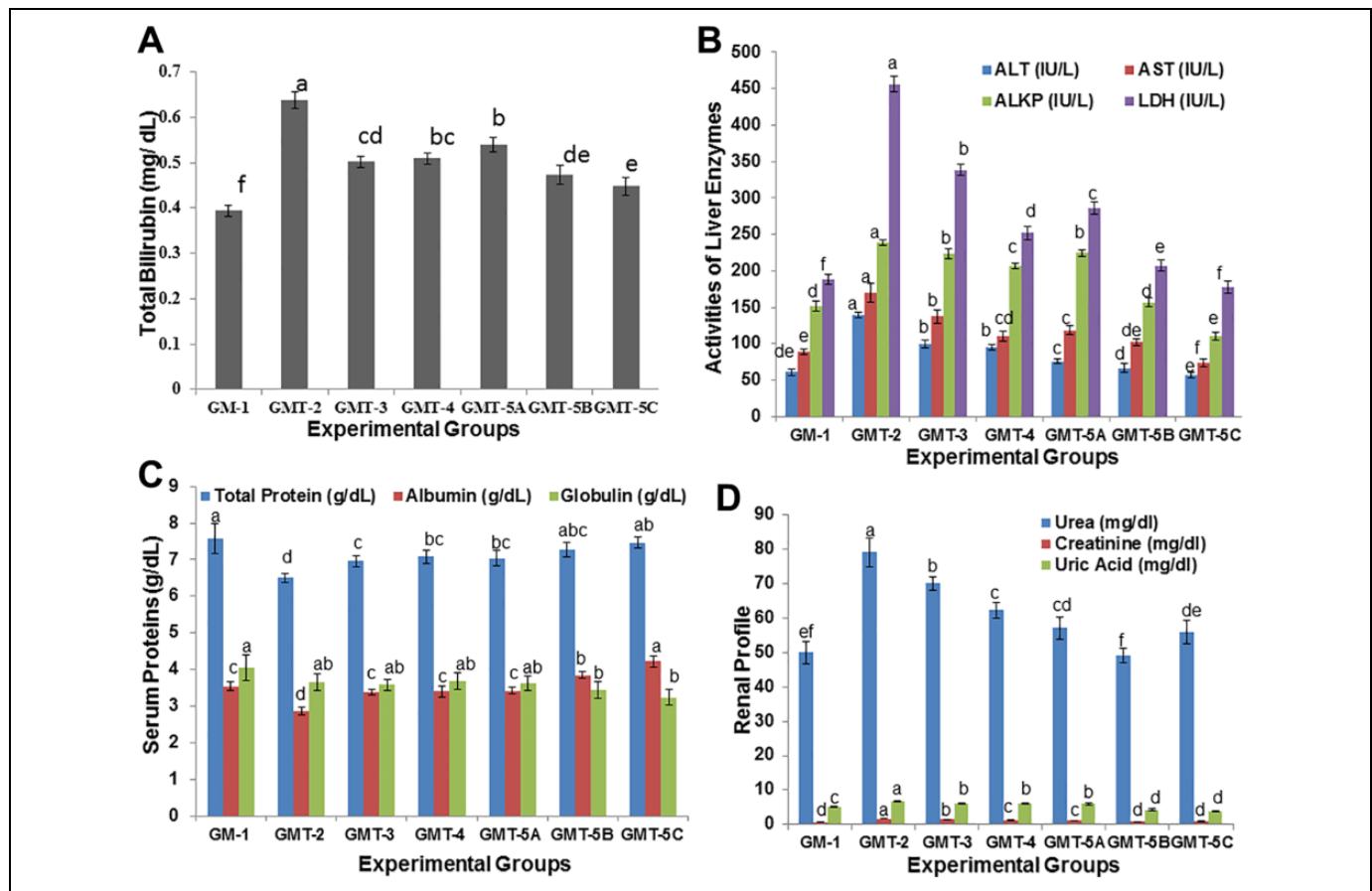


Figure 4. (A) Concentration of Serum Total Bilirubin (B) Activities of liver enzymes including alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALKP) and lactate dehydrogenase (LDH) (C) Concentration of serum proteins like Total proteins, albumin and globulin and (D) Concentration of blood urea, creatinine and uric acid in the blood samples collected from different experimental groups at the end of trial. Values are Mean \pm SEM (standard error of Mean) of the study groups. Column share the same alphabets indicate non-significant ($P > 0.05$) differences.

significantly ($P < 0.01$) decreased the body weight and reproductive hormones including testosterone, LH, FSH and prolactin while progesterone and estradiol increased significantly ($P < 0.05$) in rats in comparison with the normal rats (Table 4 and Figure 5). On the administration of different doses of hydroethanolic extracts significant ($P < 0.01$) restoration of these hormones in comparison of positive control animals administered testosterone injection and Laboob-e-Kabir have been reported (Table 4 and Figure 5).

Histological examination of testicular tissue. The histological examination of testicular tissues revealed that CCl_4 intoxication of rats significantly damaged the spermatogenic cells in the seminiferous tubules (Figure 6B) as compared to normal control group, as normal testis are composed of multiple seminiferous tubular cells with germinal epithelium (4-6 layers) at various spermatogenic stages with mature spermatozoa in the central lumen. The other cells including sertoli cells and leydig cells are also present in adequate number in normal testis (Figure 6A). The sections from both positive controls and rats administrated hydroethanolic plant extract showed noticeable

improvement in the histo-architecture after CCl_4 intoxication (Figure 6C-E).

Discussion

Phytochemical Constituents and Antioxidant Potential

A wide range of medicinal plants are being used with several therapeutic potentials due to the presence of phytochemical constituents including alkaloids, flavonoids, steroids, glycosides, tannins and saponins.⁴⁵ Natural alkaloid as well as their synthetic derivatives have proven to have wide range of pharmacological activities including antimicrobial, analgesic and antispasmodic⁴⁶ and it was reported that the antioxidant potential of herbal preparations is due to the presence of phenolic compounds.⁴⁷ Moreover, it was well reported that ethanol and methanol act as effective solvents for the extraction of antioxidant phenolic compounds¹⁵ and most important groups of natural products include flavonoids are the widespread class of natural phenolic constituents. It was reported that quenching of active oxygen species is due to several flavonoids present in extract.⁴⁸

Table 3. Effect of Selected Ethanolic *E. grandiflorum* Extract and Control Drugs on Stress Markers in Male Albino Rats.

Parameters	GM-1	GMT-2	GMT-3	GMT-4	GMT-5A	GMT-5B	GMT-5C	P-value
TAS	0.823 ± 0.051 ^{CD}	0.504 ± 0.038 ^F	0.718 ± 0.051 ^E	0.737 ± 0.044 ^{DE}	0.906 ± 0.026 ^C	1.473 ± 0.073 ^B	2.402 ± 0.037 ^A	<0.01
TOS	15.758 ± 2.055 ^{BC}	27.748 ± 2.317 ^A	24.672 ± 2.320 ^A	23.940 ± 2.336 ^A	19.544 ± 1.547 ^B	15.905 ± 1.132 ^{BC}	12.089 ± 1.278 ^C	<0.01
MDA	11.51 ± 1.55 ^C	18.28 ± 1.54 ^A	15.75 ± 1.27 ^{AB}	15.08 ± 1.17 ^B	12.33 ± 1.10 ^C	9.77 ± 0.85 ^{CD}	8.13 ± 1.31 ^D	<0.01
SOD	37.85 ± 2.74 ^{CD}	26.92 ± 1.95 ^E	32.86 ± 2.58 ^D	36.20 ± 2.69 ^{CD}	38.53 ± 2.00 ^C	47.65 ± 2.37 ^B	55.68 ± 3.06 ^A	<0.01
CAT	14.74 ± 2.07 ^{CD}	9.62 ± 1.59 ^E	11.32 ± 1.60 ^{DE}	15.54 ± 1.56 ^C	15.22 ± 1.60 ^{CD}	22.92 ± 2.23 ^B	27.85 ± 2.94 ^A	<0.01

GM-1; normal control male rats given only diet, GMT-2; toxic group male rats intoxicated with 0.5 mL 20% CCl₄, GMT-3; Positive control group male rats given 0.5 mL 20% CCl₄ + 2.5 mg/Kg bw testosterone injection, GMT-4; Positive control group male rats given 0.5 mL 20% CCl₄ + 200 mg/Kg bw Laboob e Kabir (Polyherbal Preparation) orally, GMT-5A, GMT-5B, GMT-5C; Test group male rats intoxicated with 0.5 mL 20% CCl₄ and administered with ethanolic extract *E. grandiflorum* in dose concentrations of 50, 100 & 200 mg per Kg bw, orally daily respectively TAS (Total antioxidant status), TOS (Total oxidant status), MDA (Malondialdehyde), SOD (Superoxide dismutase), CAT (Catalase) Values are mean ± SE (standard error) of means of the study groups. Different letters in superscripts in same row indicate significant group mean differences. The p < 0.05 considered statistically significant while p < 0.01 indicates highly significant.

Table 4. Effect of Ethanolic Plant Extract of *E. grandiflorum* and Control Drugs on Body Weight (g) and Testis Weight in Male Albino Rats.

Parameters	GM-1	GMT-2	GMT-3	GMT-4	GMT-5A	GMT-5B	GMT-5C	p-value
Initial body weight (g)	160.6 ± 6.95B	170 ± 6.44B	170.8 ± 8.25B	165 ± 5.79B	187 ± 6.52A	174.8 ± 9.68AB	166.2 ± 8.87B	<0.01
Final body weight (g)	248 ± 9.083BC	237.4 ± 9.274C	264 ± 7.176AB	250 ± 8.916BC	277.8 ± 6.325A	270 ± 8.396A	271 ± 8.276A	<0.01
Body weight gain (g)	87.4 ± 3.962BC	65.4 ± 4.159D	93.8 ± 4.324B	85 ± 3.050C	90.8 ± 4.324B	95.2 ± 4.637A	104.8 ± 3.317A	<0.05
Testis Weight Gain (g)	4.472 ± 0.145C	3.442 ± 0.178E	4.588 ± 0.131C	4.104 ± 0.073D	5.058 ± 0.106B	5.762 ± 0.183A	6.034 ± 0.126A	<0.05
Relative weight gain in Testis (g)	1.804 ± 0.044BC	1.455 ± 0.108D	1.740 ± 0.095BC	1.644 ± 0.073C	1.827 ± 0.047B	2.136 ± 0.100A	2.229 ± 0.101A	<0.01

GM-1; normal control male rats given only diet, GMT-2; toxic group male rats intoxicated with 0.5 mL 20% CCl₄, GMT-3; Positive control group male rats given 0.5 mL 20% CCl₄ + 2.5 mg/Kg bw testosterone injection, GMT-4; Positive control group male rats given 0.5 mL 20% CCl₄ + 200 mg/Kg bw Laboob e Kabir (Polyherbal Preparation) orally, GMT-5A, GMT-5B, GMT-5C; Test group male rats intoxicated with 0.5 mL 20% CCl₄ and administered with ethanolic extract *E. grandiflorum* in dose concentrations of 50, 100 & 200 mg per Kg bw, orally daily respectively Values are Mean ± SE (standard error) of means of the study groups. Different alphabetic letters in same row specify the significant differences in group means. The level of significance was considered at p < 0.05 while p < 0.01 indicates highly significant.

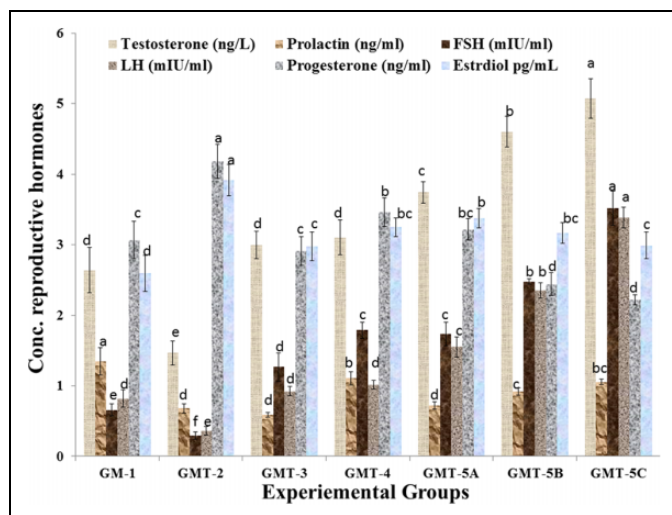


Figure 5. Concentration of reproductive hormones including testosterone, prolactin, FSH, LH, progesterone and estradiol in the blood samples collected from different experimental groups at the end of trial. Values are mean \pm SE (standard error) of means of the study groups. Column share the same alphabets indicate non-significant ($p > 0.05$) differences.

Defect in the natural antioxidant defense system might create oxidative stress which might be associated with different types of disorders and other healthcare problems⁴⁹ and to prevent these oxidant associated disorders natural antioxidants including flavonoids, carotenoids as well as vitamins like A, C and E with food supplements are used.⁵⁰

It was reported that *E. grandiflorum* has good antioxidant potential, and could act as natural antioxidants in pharmaceutical preparations, foods and cosmetics, and our results are in agreement with the findings of Yang et al.,⁵¹ who also found the good radical scavenging activity of Epimedium species. Moreover, results also explored that the increase in the reductive capacity is directly proportional to the concentration of plant extracts. In addition, it was also reported that the hydro-ethanolic extract of *E. grandiflorum* contains phyto compounds responsible for reducing potential, a significant indicator of its antioxidant capacity.^{52,53}

Hemolytic and Thrombolytic Activities

Evaluation of drugs toxicity is one of the major concerns in drug development⁴⁴ because most of the herbal preparations have a wide range of health benefits. Very high viscosity of semen is also a cause of infertility because this significantly affects the motility of spermatozoa. Although, a wide range of thrombolytic medicines are available but many of them have severe side effects which might lead to bleeding and embolism.⁵⁴ In the previous studies, it was reported that secondary metabolites present in the plant extract might be responsible for the thrombolytic activity. Fuentes et al.⁵⁵ determined that flavonoids and saponins are the phytochemicals responsible for

the thrombolytic activity of plants and these compounds have ability to dissolve the fibrin clot.

In Vivo Hydroethanolic Extract' Therapeutic Response

Hepatoprotective and renoprotective activities of *E. grandiflorum* extract. Liver as a chief organ in body plays vital roles in the body like synthetic, detoxification, storage, excretion as well as secretion; and the failure of any these metabolic process might associated with liver abnormalities leading to increased serum liver enzymes levels in blood circulation.⁵⁶ The improvement in liver enzymes of plants treated rats suggests the extract has hepatoprotective potential of Epimedium.² It was also found that Epimedium species have potential to restore the transaminases reported by Lee et al.⁵⁷

In Chinese Traditional Medicine, the kidney is alleged to be crucial for sexual and bone health. To tonify the renal functions and stimulate the yang, epimedium species are under in clinical practice and latest *in vitro* and *in vivo* pharmacological studies demonstrated that epimedium plants have a wide range of therapeutic applications including renoprotective activities.¹ Intra-gastric administration of 5 g/kg b.w daily of Epimedium to male rats, it was reported that kidney functions are restored by recovering the injured Kidney cell which also up regulate the bone formation by increasing the expression of renal hormone genes.⁵⁸

Total oxidant (TOS), antioxidant status (TAS) and stress markers in rats' blood. Antioxidant systems both enzymatic and non-enzymatic act the natural body defense mechanism to neutralize the oxidant molecules. Total antioxidant status in the body often revealed the dynamic equilibrium between the body's antioxidant defense and pro-oxidants.⁵⁹ Physiologically, body have capacity to generate enough antioxidants or are taken as food supplements for the detoxification of free radical species produced⁶⁰ and in addition, oxygen free radicals to provide the energy source produced due to the enzymatic reduction of oxygen.⁶¹ Our results are coherent with the finding of Yang et al.,⁶² who also reported the antioxidant potentials of Epimedium species in experimental rats. As *in vitro* analysis of *E. grandiflorum* for antioxidant parameters including reducing as well as scavenging potential revealed good antioxidant activities which might be due to the presence of high contents of flavonoids and phenolic compounds as their active secondary metabolites, therefore these compounds also provide good antioxidant defense system in animals after intoxication with CCl₄.⁶³

Change in body, testis weight, reproductive hormones and histo-architecture of testis. Epimedium plants not only used as medicines in Chinese Traditional medicine for the treatment of disease but also have importance to prevent disease and strengthen the body as supplement.¹ Reproductive hormones including gonadotrophins, testosterone and prolactin influence erectile processes in males.^{64,65} The reproduction is tightly regulated by hypothalamo-pituitary gonadal axis and

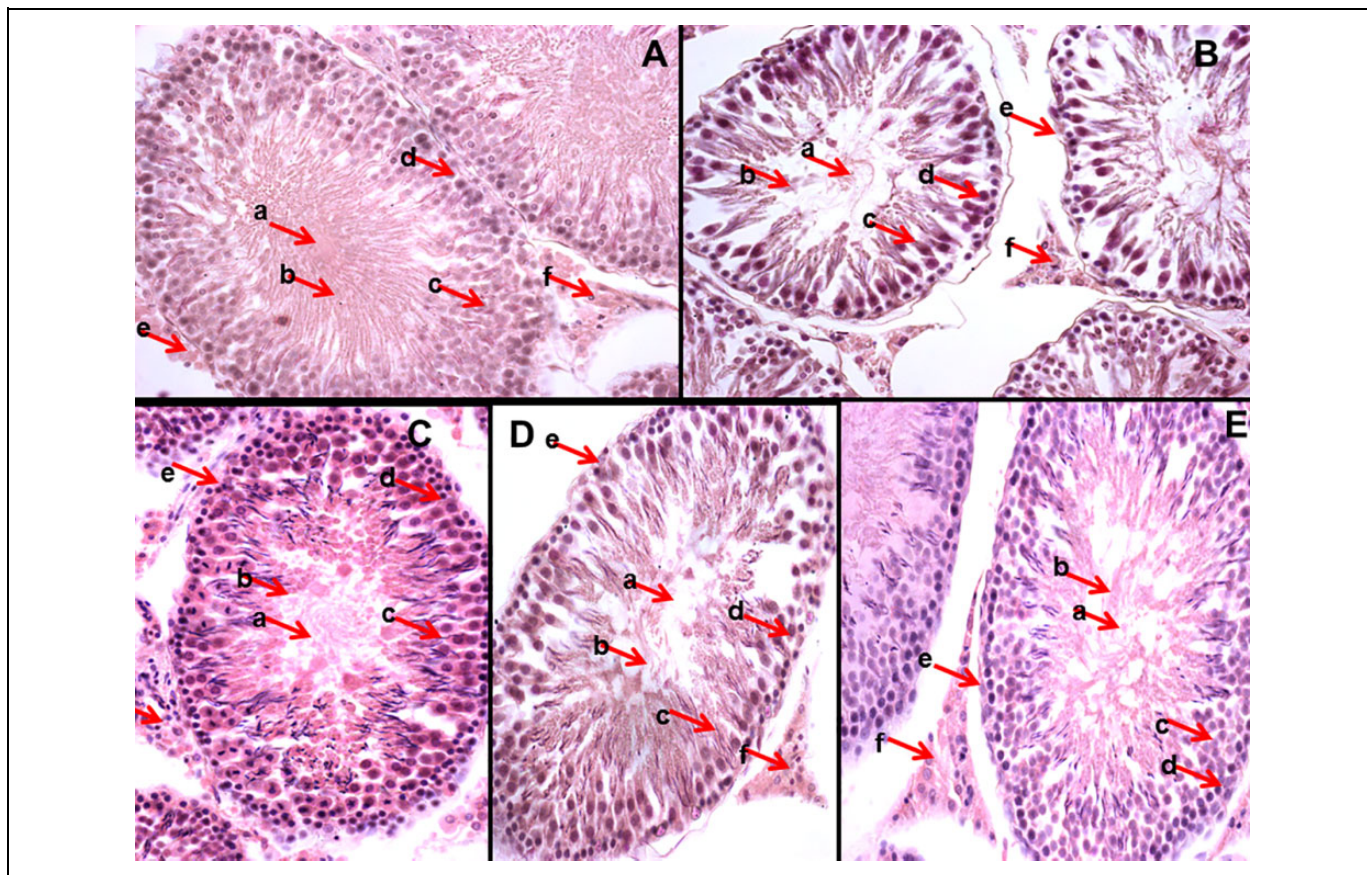


Figure 6. Histological Sections taken from Testicular tissues of different experimental male rats and stained with H & E stain (A) GM-I (B) GMT-2 (C) GMT-3 (C) GMT-4 (D) GMT-5B. The alphabets (a-f) indicates different histological features of testis as (a) represent the lumen of seminiferous tubule (b) shows mature spermatozoa (c) indicates primary spermatocyte (d) highlight the spermatogonium (e) marks the seminiferous tubules basement membrane and (f) represent the leydig cells present between seminiferous tubules spaces.

Gonadotrophin releasing hormone (GnRH) from hypothalamus influence the secretion of gonadotrophins; FSH and LH from the anterior pituitary. FSH stimulate the spermatogenesis while LH have role in the production and release of testosterone, male reproductive hormone, from the leydig cells essential for spermatogenesis and for reproductive system development like testis and prostate.⁶⁶ It also regulate the expression of secondary sexual characteristic in males like increased muscle mass, bone mass, the endocrine system and growth of body-hair.⁶⁷ FSH function is to stimulate the process of spermatogenesis while LH play role in stimulating the testosterone synthesis and release. Increased blood flow is influenced by testosterone which in turn stimulates the growth of the target tissues. The current study is in agreement with findings of Khan and Ahmed,⁶⁸ who also reported that CCl_4 induced testicular toxicity in rats and described that administration of CCl_4 caused the atrophy of testicular tissue, germinal layer degeneration, lowered the serum level of gonadotropins (FSH and LH) and testosterone in male rat.⁶⁸ The impairment in reproductive hormones might be due to the direct impact of CCl_4 on leydig cells and make them unresponsive to FSH and LH, leads to decreased secretion of testosterone. In addition, it was also reported that many hormonal fluctuations like lower level of

serum cortisol, testosterone, FSH, and insulin and impairment of prolactin secretion are associated with liver disorders in males.^{63,65} Moreover, it could be suggested that CYP17A1 enzyme action is inhibited by the action of CCl_4 during intoxication phase and in the leydig cells progesterone produced could not be metabolized to androgen which ultimately leads the decreased concentration of testosterone and increased level of progesterone. Hence the decreased level of progesterone and increased level of testosterone in treated animal groups revealed the effects of plants extract are to counter this inhibitory effect of CCl_4 .⁶⁹

Moreover, it was also find that Epimedium extracts possess a male-hormone-like effect and the administration of epimedium extract (2.0 mg/kg body weight (bw) for 2 week (14 days) orally daily significantly increased the sexual function, weight of attached genitals and improved the testosterone level in the plasma in rats.⁷⁰ Furthermore, Luo,⁷¹ investigated that glycosides from *E. grandiflorum* 5 g/kg for 8 weeks to guinea pigs significantly improved the body weight, testicular growth, spermatozoa parameters including density, viability in the epididymis and vice-testis and also increased the of the testicles, epididymis, adenohypophysis and seminal vesicles. The improvement in the sexual arousal was also reported on

administration of plant extract that might be due to improvement in the reproductive hormones.⁷¹ Our finding are also coherent in the findings of She et al., who on administration of the total flavonoids from *E. brevicornum* (150.0 mg/kg bw for 7 days) to the rats, observed significantly raised the level of reproductive hormones including testosterone, estradiol and luteinizing hormone levels as well as increased the weight of the anterior pituitary gland, epididymis and seminal vesicles in juvenile rats. Such finding revealed the *Epimedium* plants improving impact on both structural male reproductive system and reproductive endocrine activities.⁷²

It could be concluded that *E. grandiflorum* contained a wide range of phytochemical constituents, their significant *in vitro* and *in vivo* antioxidant potential, significant hepatoprotective, renoprotective and reproductive hormones restoring capacity in male albino rats as therapeutic response directly proportional to doses of hydroethanolic extract administered. However, more research is required to isolate the novel compounds from this therapeutic plant to address the problems particularly of impotency.

Acknowledgments

The authors acknowledged the technical supervision and providing the space in Clinical Biochemistry Laboratory, Department of Biochemistry, Government College University Faisalabad–Pakistan to complete this research work.


Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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