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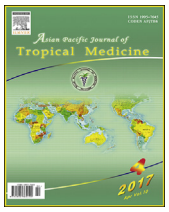

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Pistacia chinensis: Strong antioxidant and potent testicular toxicity amelioration agent

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ABSTRACT

Objectives: To evaluate *in vitro* and *in vivo* antioxidant potency of *Pistacia chinensis* (*P. chinensis*) bark and leaves extracts along with its protective role against CCl_4 induced toxicity in testis of the rat.

Methods: Various *in vitro* models such as DPPH, ABTS, hydrogen peroxide, superoxide, hydroxyl and nitric oxide scavenging activities, anti-lipid peroxidation activity, phospho-molybdenum activity, β carotene bleaching assay was used for analysis of antioxidant potential. Experimental groups for *in vivo* study were: Group Ⅰ (control) untreated, Group Ⅱ (Vehicle control), Group Ⅲ (1 mL/kg b.w 30% CCl_4), Group Ⅳ (1 mL/kg b.w CCl_4 + Silymarin), Group Ⅴ (200 mg/kg b.w PCBE + CCl_4), Group Ⅵ (400 mg/kg b.w PCBE + CCl_4) and Group Ⅶ (400 mg/kg b.w PCBE alone).

Results: *In vitro* antioxidant assays displayed significant results and the highest activity was not specified to a specific extract. However, ethyl acetate extract of bark (PCBE) showed highest results in most of the antioxidant assays *i.e.* beta-carotene bleaching, hydroxyl radical scavenging, ABTS, lipid peroxidation and superoxide radical scavenging activity. On this base, this fraction was selected for *in vivo* antioxidant experiment. Testis tissues were analyzed to observe the protective effects of PCBE on antioxidant enzymes; catalase, superoxide dismutase, peroxidase, glutathione-S-transferase, glutathione reductase, glutathione peroxidase and quinone reductase activities and glutathione (GSH) as well as nitrite content. Profile of plasma testosterone was also compared to various treatments. Observation suggests a protective role of *P. chinensis* against CCl_4 induced toxicity.

Conclusions: It is concluded that some bioactive antioxidants of *P. chinensis* bark might be a good source to isolate the potent antioxidant components.

1. Introduction

Oxidative stress occurs as an outcome of an imbalance between the formations of reactive oxygen species (ROS) and the available antioxidant defense against them. ROS are produced consistently by many physiologic and metabolic processes [1,2]. Spermatogenesis is an extremely coordinated process which takes place in the seminiferous tubules of the testis [3]. Spermatozoa are very sensitive to ROS as their plasma membrane is comprised of polyunsaturated fatty acids (PUFA), which can be easily oxidized and they also lack cytoplasm to produce a robust preventive and repair mechanism against ROS [4].

Although carbon tetrachloride (CCl_4) is known to be hepatotoxic as well as nephrotoxic to humans and experimental animals. It is still being used in the fumigation of grains, in dry cleaning, in filling fire extinguishers, and as an insecticide. The mechanism of CCl_4-induced liver injury is well studied in the rat model. Accidental ingestion of CCl_4 results in the accumulation of CCl_4 in the testes and causes damage to this organ. Testes have a great affinity for CCl_4 and contain cytochrome P450 which activates converse of CCl_4 to toxic metabolites. The initial step in the tissue injury induced by CCl_4 is its cytochrome

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P450-mediated formation of trichloromethyl radical (CCl_3) and trichloromethyl peroxyl (CCl_3OO) free radicals. The over-production of CCl_3 free radicals initiates membrane lipid and protein oxidation, eventually leading to various pathological changes. Free radical reactions have been implicated in the pathology of many disease conditions like atherosclerosis, ischemic heart diseases, the aging process, inflammation, diabetes, immunosuppression, and neurodegenerative disease. Inadequate antioxidant defenses lead to disturbances in redox homeostasis causing damage to lipids, proteins, carbohydrates, and DNA. Exogenous antioxidants such as polyphenolic compounds in medicinal plants may constitute an antioxidative defense by scavenging free radicals and possibly increase the longevity of biological systems in such conditions [5].

In the recent scientific progress throughout the world, the medicinal qualities of the plants have been studied due to their fewer side effects, potent antioxidant activities and commercial viability [6]. Phenolics and flavonoids compounds of plants show manifold biological benefits by exerting antioxidative scavenging role [7].

The present study aim was to determine the antioxidant potential of *Pistacia chinensis* (*P. chinensis*) plant by using multiple *in vitro* antioxidant assays and *in vivo* models. *In vitro* studies were conducted to evaluate the antioxidant capacity of the leaves and bark. The potent antioxidant component was evaluated by *in vivo* experiment against CCl_4 induced toxicity in rat testis.

2. Materials and methods

2.1. Plant collection and extraction

A collection of *P. chinensis* plant leaves and bark samples were carried out from the surroundings of Quaid-i-Azam University, Islamabad in September 2012. The identification of the sample was carried out at Herbarium of Pakistan, Quaid-i-Azam University Islamabad. The plant leaves and stem bark were chopped into pieces, removed the dust particles and then shade dried at 25 °C for about two weeks. To prepare the dried powder of sample Willy Mill of 60-mesh size was used and a then powdered sample of leaves and bark were used separately for solvent extraction. One kg powder sample of leaves and bark was extracted two times with 2 L of crude methanol at 25 °C for 48 h. Filtration was carried out using Whatman No. 1 filter paper and rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) was used to concentrate the filtrate under reduced pressure at 40 °C. The same procedure was repeated to obtain the ethanol extract of each sample by using 80% ethanol instead of methanol. Both the extract were stored at 4 °C for *in vitro* and *in vivo* experiments.

2.2. In vitro antioxidant assays

2.2.1. 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity

The methodology of Sirajuddin [8] was followed to measure DPPH radical scavenging of methanol extract of *P. chinensis*. DPPH solution was prepared by mixing 12.5 mg of DPPH in 50 mL of methanol. The optical density of stock solution was measured at 517 nm. The absorbance of 0.9 was maintained by diluting the DPPH solution if needed with methanol. An aliquot of 100 μL of a test sample of different concentrations with (30–500) μg/mL prepared in methanol was added to 1 mL of DPPH solution. After vigorous shaking Eppendorf tubes were incubated at 37 °C for 20 min. The decrease in absorbance was determined at 517 nm. With the help of following formula of Sirajuddin [8], free radical activity was determined;

$$\text{DPPH scavenging activity}(\%) = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

2.2.2. Superoxide radical scavenging activity

Superoxide radical scavenging activity was carried out following the method of Saeed, Khan [9]. An aliquot of 150 μL of test sample of different concentrations with (30–500) μg/mL methanol was added to 250 μL of 50 mM potassium phosphate buffer (pH 7.6), 150 μL of 50 mM riboflavin, 126 μL of 20 mM PMS (potassium persulphate), 50 μL of 0.5 mM NBT (nitro blue tetrazolium). The reaction mixture was illuminated with a fluorescent lamp for 30 min to start a reaction. Using spectrophotometer optical density was determined at 560 nm. Ascorbic acid was used as a standard. Using the following formula [9], superoxide anion scavenging activity was determined;

$$\text{Super oxide scavenging activity}(\%) = \left(1 - \frac{\text{Abs Sample}}{\text{Abs Control}}\right) \times 100$$

2.2.3. Hydroxyl radical scavenging activity

The methodology of Halliwell, Gutteridge [11] was adopted to measure the hydroxyl radical scavenging activity of extracts of *P. chinensis*. An aliquot of 100 μL of test sample of different concentrations with (30–500) μg/mL prepared in methanol added to 100 μL of 100 mM $FeCl_3$ and 100 mM EDTA (1:1 ratio), 50 μL of 200 mM H_2O_2 and 250 μL of 2.8 mM 2-deoxyribose (prepared in 50 mM phosphate buffer). Fenton reaction was initiated by adding 100 μL of ascorbate (300 mM). After incubation for 1 h at room temperature, 500 μL of 1% thiobarbituric acid (prepared in 50 mM NAOH) and 500 μL of 2.9% TCA was added and reaction tubes were boiled on a water bath for 10 min. Optical density was determined at 532 nm after cooling of the reaction solution. Following formula [10] measured the hydroxyl radical scavenging activity;

$$\text{Hydroxyl radical scavenging activity}(\%) = \left(1 - \frac{\text{Abs Sample}}{\text{Abs Control}}\right) \times 100$$

2.2.4. H_2O_2 scavenging activity

The methodology of Shah, Khan [11] was followed to measure hydrogen peroxide scavenging activity of extracts of *P. chinensis*. An aliquot of 100 μL of a test sample of different concentrations with (30–500) μg/mL prepared in methanol was added to 300 μL of 50 mM phosphate buffer and 600 μL of 2 mM H_2O_2 (prepared in 50 mM phosphate buffer). Mixing was done by shaking and placed at room temperature for 15 min. Optical density observed at 230 nm using phosphate buffer as a blank. By following formula [12], scavenging of H_2O_2 activity was determined;

$$\text{Hydrogen peroxide scavenging activity}(\%) = \left(1 - \frac{\text{Abs Sample}}{\text{Abs Control}}\right) \times 100$$

$$\text{Lipid peroxidation activity}(\%) = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

2.2.5. 2, 2′-Azino-bis[3-ethylbenzothiazoline-6-sulphonic acid] (ABTS) radical scavenging activity

ABTS radical scavenging of *P. chinensis samples* was determined by Saeed, Khan [9]. ABTS radical cations were generated by preparing ABTS solution (by mixing 3.5 mM ABTS and 1.2 mM potassium persulphate and incubating in dark for at least 12 h). The optical density of ABTS solution was measured at 734 nm and absorbance was set at 0.7 by diluting with 70% ethanol. An aliquot of 100 μL of a test sample of different concentrations with (30–500) μg/mL prepared in methanol was added to 1 mL of ABTS solution. Change in absorbance was noted for 6 min. The decrease in absorbance was observed at 517 nm. With help of following formula [9], percentage inhibition of ABTS radical cation formation was determined;

$$\text{Inhibition}(\%) = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

2.2.6. β-carotene assay

The methodology Sahreen, Khan [13] was followed to measure beta-carotene bleaching of extracts of *P. chinensis*. The β-carotene emulsion was prepared by mixing 1 mg of β-carotene in 5 mL of chloroform and addition of 25 μL of linoleic acid and 200 μL of Tween 20. After chloroform evaporation 25 mL of distilled water was added. An aliquot of 100 μL of a test sample of different concentrations with (30–500) μg/mL prepared in methanol was added to 1 mL of β-carotene emulsion. Optical density was measured at 470 nm and reaction mixture was incubated at 45 °C for 2 h. With the help of following formula [13], percentage inhibition of β-carotene bleaching was determined;

$$\text{Inhibition}(\%) = \frac{\text{Abs after 2 hours}}{\text{Abs Initial}} \times 100$$

2.2.7. Lipid peroxidation assay

Lipid peroxidation was determined by following the method of Shah, Khan [11]. In this method, egg yolk homogenate was used to prepare the lipid peroxide as lipid rich media to measure the lipid peroxide formed as lipid rich media. 10% egg yolk homogenate was prepared in 1.15% KCl solution and ultracentrifuged for 15 min. Egg homogenate 0.5 mL was mixed with 0.1 mL sample in a test tube and raised the volume by adding distilled water up to 2 mL. Then 0.05 mL of $FeSO_4$ (0.07 M) was mixed and lipid peroxidation induced by 30-min incubation. After that 0.8% TBA (w/v), 1.5 mL (prepared in 1.1% sodium dodecyl sulfate), 20% acetic acid, 1.5 mL of (pH 3.5) along with 20% TCA 0.05 mL were mixed well, vortexed and suspended for 60 min in a boiling water bath. Finally, 5.0 mL of *n*-butanol was added to each tube and centrifuged for 10 min at 3000 rpm. Pinkish chromogen was produced and absorbance was measured at 532 nm. Percentage inhibition was calculated using formula [11];

2.2.8. Nitric oxide scavenging assay

The methodology of Balakrishnan, Panda [14] was adopted to measure the nitric oxide scavenging activity of *P. chinensis* extract. Griess reagent was used to measure the nitric oxide activity. An aliquot of 100 μL of a test sample of different concentrations with (30–500) μg/mL prepared in methanol was added to 100 μL of 10 mM sodium nitroprusside solution (prepared in phosphate buffer saline). After incubation of 3 h at room temperature, 1 mL of Griess reagent was added and Optical density was measured at 546 nm using the standard of ascorbic acid. With the help of following formula [15], percentage inhibition of ABTS radical cation formation was determined;

$$\text{Nitric oxide activity}(\%) = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

2.3. In vivo study

2.3.1. Animals and experimental design

P. chinensis ethanolic bark extract exhibiting potent *in vitro* antioxidant activity in most of the *in vitro* assays was evaluated against CCl_4 induced toxicity in testis in Sprague–Dawley male rats. Forty-two Sprague–Dawley male rats with (150–200) g were donated by National Institute of Health (NIH) Islamabad and kept at room temperature in steel cages under 12-h dark/light cycle. The study protocol was approved by the ethical board of Quaid I Azam University, Islamabad.

Group Ⅰ was treated as control while Group Ⅱ was treated as vehicle control. Group Ⅲ was given 30% CCl_4 intraperitoneally in olive oil at the dose of 1 mL/kg b.w; on alternate days. Group Ⅳ received 200 mg/kg b.w of silymarin as standard chemical on alternate days following one hour after CCl_4 administration. Group Ⅴ and Ⅵ were administered orally with PCBE extract 200 and 400 mg/kg b.w after one hour of CCl_4 treatment on alternate days. However, Group VII was given PCBE at the dose rate of 400 mg/kg b.w on alternate days.

2.3.2. Dissection of animals

Dissection was carried out after on completion of eight weeks of experiment duration. Treatment was stopped 24 h before the dissection. Falcon tubes were used for blood collection and serum was separated by centrifugation and stored in the refrigerator. After blood collection, the testis was dissected out where and washed in cold saline. Blotting paper was used for drying the saline wet organs. The tissues after drying were separated into two portions. For histology, one testis from each animal was preserved in fixative sera; while for biochemical studies the tissue was treated with liquid nitrogen and stored at −70 °C.

2.4. Estimation of antioxidant enzymes

A total of 100 mg of testis organ tissue was ground in 1 mL of 100 mM Potassium phosphate buffer having 1 mM EDTA at pH 7.4 thus homogenate was prepared. Clean falcon tubes were used for supernatant collection after centrifugation at 12000 ×*g*

at 4 °C for 30 min and were used for antioxidant enzymes analysis.

2.4.1. Catalase activity

The extent of H_2O_2 decomposition measured the CAT activity and protocol of Chance and Maehly [16] was followed. Twenty-five μL of tissue homogenate was mixed with 5.9 mM H_2O_2 (100 μL) and 50 mM Potassium phosphate buffer (625 μL) at pH 5.0. The disappearance of H_2O_2 in the reaction mixture by catalase was measured at 240 nm spectrophotometrically. One unit CAT activity is defined as units/min change in absorbance of 0.01.

2.4.2. Peroxidase activity

The method of Chance and Maehly [16] was followed to measure the POD activity spectrophotometrically with slight change. A total of 25 μL tissue homogenate was mixed to 75 μL of 40 mM H_2O_2, 625 μL of 50 mM Potassium phosphate buffer (pH 5.0) and 25 μL of 20 mM guaiacol. Absorbance per minute change was measured at 470 nm. One unit POD activity is defined as units/min change in absorbance of 0.01.

2.4.3. Superoxide dismutase activity

SOD activity was measured by the method of Kakkar, Das [17] using phenazine methosulphate and sodium pyrophosphate buffer. Centrifugation of tissue homogenate was carried out at 1 500 ×*g* for 10 min followed by 10 000 ×*g* for 15 min. SOD activity was determined from the collected supernatant. A total of 150 μL of supernatant was mixed with 50 μL of 186 μM phenazine methosulphate and 600 μL of 0.052 mM sodium pyrophosphate buffer (pH 7.0). To start enzymatic reaction 100 μL of 780 μM NADH was also added. After 1 min the reaction was stopped by the addition of 500 μL of glacial acetic acid. Color intensity was measured by determining optical density spectrophotometrically 560 nm. Results are showed in units/mg protein.

2.4.4. Reduced glutathione activity

Protocol of Khan, Khan [18] was followed to measure GSH activity. Precipitation of 500 μL of tissue homogenate was carried out by the addition of 500 μL of (4%) sulfosalicylic acid. samples were centrifuged at 1 200 ×*g* after 1-h incubation at 4 °C for 20 min. A total of 33 μL of collected supernatant was added to a mixture containing 900 μL of 0.1 M potassium phosphate buffer (pH 7.4) and 66 μL of 100 mM DTNB, which formed yellow colored complex reduced glutathione. Absorption at 412 nm was measured as μM GSH/g tissue. It represents the GSH activity.

2.4.5. Glutathione peroxidase activity

The reaction mixture of glutathione-S-transferase activity consisted of 1.475 mL phosphate buffer (0.1 M, pH 6.5), 0.2 mL reduced glutathione (1 mM), 0.025 mL (CDNB; 1 mM) and 0.3 mL of tissue homogenate in a total volume of 2 mL. The changes in the absorbance were recorded at 340 nm and enzymes activity was calculated as nM CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6×10^3/M/cm [19].

2.4.6. Quinone reductase activity

Quinone reductase activity was determined by the method of Khan, Khan [20]. Reduction of dichlorophenol indophenol complex measured the quinone reductase activity. Tissue homogenate aliquot of 33.3 μL was added to 6.6 μL of 0.1 mM NADPH, 233 μL of bovine serum albumin, 33.3 μL of 50 mM FAD and 710 μL of 25 mM tris–HCL buffer (pH 7.4). Optical density measured with a spectrophotometer at 600 nm. QR activity was calculated as nmoles of DCPIP reduced/min/mg protein using molar extinction coefficient of 2.11×10^4/M/cm.

2.4.7. Glutathione reductase activity

GSR activity was determined by the method of Ahmad, Khan [21]. In GR activity NADPH is used as the substrate. 50 μL of tissue homogenate was added to 50 μL of 0.1 mM NADPH, 50 μL of 0.5 mM EDTA, 825 μL of 0.1 M sodium phosphate buffer (pH 7.6), 25 μL of 1 mM oxidized glutathione and NADPH decomposition is measured with a spectrophotometer at 340 nm. GSR activity was determined as the amount of NADPH oxidized/min/mg protein using molar extinction coefficient of 6.23×10^3/M/cm.

2.4.8. Glutathione-S-transferase activity

GST assay was based on the formation of CDNB conjugate. An aliquot of 150 μL of tissue homogenate was added to 100 μL of 1 mM reduced glutathione, 12.5 μL of 1 mM CDNB and 720 μL of sodium phosphate buffer. Optical density was recorded at 340 nm. Using a molar coefficient of 9.61×10^3/M/cm, GST activity was measured as the amount of CDNB conjugate formed/min/mg protein [21].

2.4.9. Lipid peroxidation assay (TBARS)

TBARS (thiobarbituric acid reactive substances) of tissue homogenate were assessed by using TBA. Tissue homogenate 100 μL was mixed to 100 μL of ascorbic acid (100 mM), 290 μL of sodium phosphate buffer (pH 7.4). Above reaction mixture was incubated in a shaking water bath for 1 h at 37 °C. Ten percent TCA was added (500 μL) to stop the reaction. A total of 500 μL with 0.67% TBA was mixed and reaction tubes were put for 15 min in boiling water bath. Tubes were shifted on the crushed ice after 15 min for 5 min and centrifuged at 2 500 ×*g* for 10 min. The absorbance of the supernatant was read at 535 nm. Lipid peroxidation activity was measured using molar extinction coefficient of 1.560×10^5/M/cm as an amount of TBARS formed/min/mg tissue [11].

2.4.10. Nitrite assay

Nitrite assay was carried out by using Griess reagent. Deproteinization of tissue samples was performed by centrifugation of 100 mg of tissue at 6 400 ×*g* for 20 min with 100 μL of 0.3 M NaOH and 100 μL of 5% $ZnSO_4$. One mL of Griess reagent was added to 20 μL of the supernatant. The color change was measured at 540 nm as nitrite concentration in tissue samples using sodium nitrite standard curve [11].

2.5. Testosterone determination

For measuring testosterone level of rat serum 'Astra Biotech kit' acquired from Immunotech Company was used. It is a modest radioimmunoassay. Testosterone of samples and horseradish peroxidase (HRP) labeled testosterone binds until equilibrium occurs between them to the antibodies coated wells. The amount of testosterone is in inverse relation to the amount of

bound conjugate. Fifty μL of calibrators (0–5), Control and samples in duplicates were added into the separate wells; two wells A1–A2 were kept empty for blank. Then into each well, 150 μL of the conjugate was dissolved, except wells A1-A2. Strips were incubated for 90 min at room temperature while shaking (500–800) rpm. After these four times washing was done. Further in every individual well 100 μL of the substrate was added; (15–30) min incubation was given at room temperature in the dark, depending on the color intensity. In the same sequence as used for distributing TMB substrate at the same speed, mixing of 100 μL of stop solution into each well was done. Within 20 min absorbance was checked at 450 nm. By creating standard curve results were deliberated.

2.6. Determination of total protein concentration

For measuring total protein concentration AMP diagnostics company kit was used. In alkaline solution, in the presence of copper salt a colored complex was detected (biuret method). An aliquot of 10 μL standard (albumin) was added to 1 mL of a reagent having sodium hydroxide, copper sulfate, potassium iodide and potassium sodium tartrate. After incubation for 10 min at 37 °C, absorbance was detected at 550 nm spectrophotometrically. Distilled water was used as a blank.

Total protein calculation was done by given formula;

$$\text{Total protein concentration (mg/dL)} = \text{abs sample/abs standard} \times \text{n}$$

n = concentration of standard (mg/dL).

2.7. Statistical analysis

Computerized Graph pad prism software was used to analyze the *in vitro* data and determine IC_{50} values. However, the parameters of *in vivo* studies were analyzed with one-way analysis of variance through statistix 8.1.

3. Results

3.1. In vitro antioxidant activity evaluation of various extracts of P. chinensis leaves and barks

Various *in vitro* antioxidant assays were performed for preliminary antioxidant activity assessment of various *P. chinensis* extracts of leaves and bark. Following results were obtained.

3.1.1. DPPH free radical scavenging activity

DPPH (1, 1-diphenyl 1-2-picryl-hydrazyl) a free radical is extensively used for assessment of *in vitro* antioxidant scavenging activities of medicinal plants because it has the ability to scavenge electron from the antioxidant. Table 1 shows the IC_{50} values of *P. chinensis* plant extracts for DPPH free radicals. Among the extracts with different solvents, the PCLE and PCBM showed IC_{50} values of (39.4 ± 1.7) μg/mL and (48.3 ± 0.6) μg/mL, respectively. IC_{50} values of DPPH free radical scavenging for PCLM and PCBE were (54.46 ± 0.80) μg/mL and (45.2 ± 1.1) μg/mL, respectively. All the extract showed significantly different IC_{50} values. PCLE showed highest DPPH radical scavenging activity of near to reference ascorbic acid but significantly different ($P < 0.05$).

3.1.2. Hydrogen peroxide radical scavenging activity

The hydrogen peroxide interacts with many biologically active molecules by traversing the cell membrane at particular sites, damaging the cell and thus results in cell death. Therefore for the protection of the living system, it is compulsory to scavenge H_2O_2. Table 1 shows the IC_{50} of *P. chinensis* plant extracts against H_2O_2 scavenging activity with ascorbic acid as a standard. Lowest IC_{50} value was showed by PCLE *i.e.* (21.9 ± 1.5) μg/mL; followed by PCLM (33.1 ± 1.9) μg/mL < PCBE (35.9 ± 0.6) μg/mL < PCBM (37.23 ± 0.50) μg/mL. Narrow difference was observed with standard but significantly different.

3.1.3. ABTS radical cations scavenging activity

ABTS (2, 2 azobis-3-ethylbenzothiozoline-6-sulphonic acid) radical scavenging activity of each extract and standard compound *i.e.* ascorbic acid was determined in the assay. Each extract shows different scavenging activity against ABTS free radical cation. PCBE showed highest ABTS free radical scavenging activity (124.0 ± 2.6) μg/mL while PCLM showed lowest scavenging activity (233.0 ± 5.1) μg/mL. The other two extract showed scavenging activity in the order of PCLE (205.0 ± 9.7) μg/mL < PCBM (222.0 ± 5.1) μg/mL. The ABTS radical cation scavenging activity of ascorbic acid was (64.8 ± 1.2) μg/mL. No significant difference was observed among both type of bark extract.

3.1.4. β-carotene bleaching assay

Scavenging capacity of the various extract of *P. chinensis* plant was assessed by the β-carotene bleaching assay. β-carotene absorbance was observed to be decreased on the administration

Table 1
Antioxidant effect (IC_{50}) of various *P. chinensis* plant extracts via different *in vitro* antioxidant assays.

Activity	PCLE	PCLM	PCBE	PCBM	Standard
DPPH radical scavenging	39.4 ± 1.7d	54.4 ± 0.8a	45.2 ± 1.1c	48.3 ± 0.6b	27.8 ± 1.1e
Hydrogen peroxide scavenging	21.9 ± 1.5e	33.1 ± 1.9d	35.9 ± 0.6c	37.2 ± 0.5b	18.7 ± 0.6a
ABTS radical scavenging	205.0 ± 9.7a	233.0 ± 5.1bc	124.0 ± 2.6d	222.0 ± 5.1b	64.8 ± 1.2e
β-carotene bleaching	202.1 ± 10.2a	83.4 ± 2.2c	15.3 ± 1.4e	155.3 ± 3.4b	22.8 ± 0.1d
Superoxide radical scavenging	120.0 ± 2.0c	187.0 ± 3.1be	97.4 ± 1.3d	236.0 ± 2.1a	51.2 ± 2.3d
Hydroxyl radical scavenging	234.0 ± 8.1c	412.0 ± 11.1b	156.0 ± 1.5d	478.0 ± 15.0a	54.4 ± 0.9e
Lipid peroxidation	198.0 ± 12.1	257.0 ± 2.6a	108.0 ± 1.1d	145.0 ± 1.1c	43.1 ± 0.8e
Nitric oxide	41.3 ± 2.6b	53.7 ± 3.5a	29.00 ± 0.64c	9.24 ± 1.30e	16.1 ± 0.1d

Each value is represented as mean ± SD ($n = 3$); Data with different superscript letters indicate significant differences at $P < 0.05$ in each column; PCLE: *P. chinensis* leaves ethanol extract; PCBE: *P. chinensis* bark ethanol extract; PCLM: *P. chinensis* leaves methanol extract; PCBM: *P. chinensis* bark methanol extract; Standard: ascorbic acid.

of extracts at (50–250) μg/mL or standard antioxidants such as BHT. On the addition of extracts of *P. chinensis* and reference chemical, it inhibited oxidation of the linoleic acid and bleaching of β-carotene subsequently. PCBE showed the lowest values (15.3 ± 1.4) μg/mL needed to inhibit the 50% β-carotene bleaching. Results showed that PCBE was even lowest to reference compounds (22.8 ± 0.1) μg/mL. The other two extracts of *P. chinensis* also shows noticeable inhibitory effects of β-carotene bleaching with the IC_{50} order; PCBE < PCLM < PCBM < PCLE. The PCBE was the best and effective extract to inhibit the β-carotene bleaching linoleic acid showing significantly lowest IC_{50} than standard.

3.1.5. Superoxide radical scavenging activity

Reactive oxygen species generate superoxide radical, which produce toxic and very harmful effects to cellular components and thus are the main cause of fatal diseases. Table 1 shows superoxide radical scavenging capacity of the four extracts of *P. chinensis* plant. PCBE extract possessed the effective superoxide radical scavenging activity with IC_{50} value of (97.0 ± 1.3) μg/mL followed by PCLE (120.0 ± 2.0) μg/mL < PCLM (187.0 ± 3.1) μg/mL and <PCBM (236.0 ± 2.1) μg/mL. The scavenging effect of reference ascorbic acid was (51.0 ± 2.3) μg/mL. All the extracts showed significantly different IC_{50} range even with standard drug used.

3.1.6. Hydroxyl radical scavenging activity

Among the free radicals of oxygen, hydroxyl radical is the most toxic and thus induces various severe alterations in biomolecules. The hydroxyl radical scavenging capacity of *P. chinensis* plant extracts and antioxidants compounds used as a reference (ascorbic acid and gallic acid) are shown in Table 1. The order of plant extracts against hydroxyl radicals scavenging activity in term of IC_{50} value was as: PCBE (156.0 ± 1.5) μg/mL < PCLE (234.0 ± 8.1) μg/mL < PCLM (412.0 ± 11.1) μg/mL < PCBM (478.0 ± 15.0) μg/mL. The scavenging activity of ascorbic acid was noted (54.0 ± 0.9) μg/mL with statistically significant difference from all extracts.

3.1.7. Lipid peroxidation assay

Lipids peroxides take part in many pathological reactions, including metabolic disorders, inflammation, cellular aging and oxidative stress. The antioxidant capacity of various extracts of *P. chinensis* and reference ascorbic acid are shown in Table 1. The lowest IC_{50} value was marked for PCBE (108.0 ± 1.0) μg/mL and followed by PCBM (145.0 ± 1.1) μg/mL < PCLE (198.0 ± 12.1) μg/mL < PCLM (257.0 ± 2.6) μg/mL. The PCBE extract was most active (145.0 ± 1.1) μg/mL in inhibition of Lipids peroxidation.

3.1.8. Nitric oxide scavenging

Sodium nitroprusside in aqueous solution suddenly produces nitric oxide, which on interaction with oxygen to induce nitrite ions that can be measured by Gries's reagent. Scavengers of nitric oxide compete with oxygen, results in the decreased generation of nitrite ions. Overall the highest nitric oxide scavenging activity was depicted by PCBM (IC_{50}: 9.24 ± 1.30) μg/mL followed by PCBE (29.00 ± 0.64) μg/mL > PCLE (41.32 ± 2.60) μg/mL > PCLM (53.73 ± 3.50) μg/mL. PCBM showed lowest IC_{50} value then the standard ascorbic acid (16.0 ± 0.1) μg/mL.

Table 2
Effects of PCBE on testis CAT, POD and SOD activity, effects of PCBE on GST, GP-x, GSH and QR activity and effects of PCBE on testis protein, TBARS and nitrite activity in rat.

Treatment	CAT(U/min)	POD (U/min)	SOD (U/mg protein)	GST*	GPx*	GR*	GSH*	QR*	Protein (μg/mg tissue)	TBARS (nM/min/mg)	Nitrite (μM/mL)	Testosterone level
Control	65.38 ± 6.60d	9.96 ± 0.15d	2.93 ± 0.10e	160.2 ± 1.1e	126.5 ± 1.1d	191.1 ± 1.1e	23.3 ± 1.1d	102.8 ± 0.25e	2.75 ± 0.01b	2.89 ± 0.01g	53.20 ± 0.20c	3.23 ± 0.01b
Olive oil	65.27 ± 6.71d	10.5 ± 0.22d	2.95 ± 0.05e	163.0 ± 5.2e	128.3 ± 2.8d	192.7 ± 3.5e	26.1 ± 1.3e	98.12 ± 1.0d	2.70 ± 0.02c	2.94 ± 0.01f	51.50 ± 0.15d	3.31 ± 0.01a
CCl_4	5.06 ± 4.70a	4.06 ± 0.21a	0.92 ± 0.10a	78.00 ± 1.0a	9.330 ± 1.3a	78.00 ± 0.7a	8.00 ± 0.9a	56.30 ± 1.5a	0.70 ± 0.01g	6.13 ± 0.02a	63.70 ± 0.10a	0.95 ± 0.01g
CCl_4 + Silymarin	58.87 ± 1.02c	9.85 ± 0.05c	2.75 ± 0.05d	150.1 ± 2.0d	76.58 ± 5.8c	184.3 ± 4.0d	23.1 ± 0.7d	100.1 ± 1.0e	2.88 ± 0.01a	3.11 ± 0.01c	55.10 ± 0.35b	3.12 ± 0.01c
200 mg/kg PCBE + CCl_4	34.10 ± 5.80b	6.03 ± 0.40b	1.71 ± 0.03b	115.3 ± 2.0b	43.51 ± 3.3b	166.7 ± 10b	12.6 ± 1.2ab	86.1 ± 0.07bc	1.50 ± 0.02f	4.23 ± 0.01b	43.70 ± 0.10f	2.17 ± 0.01f
400 mg/kg PCBE + CCl_4	49.71 ± 7.91b	7.46 ± 0.50b	2.43 ± 0.32b	128 ± 6.6bc	64.21 ± 5.1c	180.3 ± 2.0c	18.1 ± 1.3c	85.12 ± 3.1b	2.40 ± 0.04d	3.08 ± 0.02d	49.80 ± 0.30e	2.87 ± 0.01e
400 mg/kg PCBE	53.00 ± 0.93d	6.56 ± 0.41d	2.53 ± 0.21d	121.7 ± 1.5d	57.00 ± 4.9b	180.7 ± 2.0c	15.4 ± 1.2bc	88.61 ± 2.5c	2.30 ± 0.02e	2.97 ± 0.01e	51.80 ± 1.10cd	2.93 ± 0.01d

Each value is represented as mean ± SD ($n = 6$); Data with different superscript letters indicate significant differences at $P < 0.05$ in each column; PCBE: *P. chinensis* bark ethanol extract; *units are expressed as n/min/mg/protein.

3.2. Testicular toxicity and protective effect of PCBE extract

Pharmacological evaluation of PCBE against CCl_4 induced toxicity in testis, antioxidant level, hormonal study and histological modifications were studied.

3.2.1. Effects PCBE on testis POD, CAT, and SOD activity in rat testis

Table 2 shows antioxidant mode of enzymes in seven groups of experiment. Dismutation of superoxide radicals occurs in SOD and conversion of hydrogen peroxide into water occurs in CAT and POD thus prevents oxidative stress in cells. CCl_4 significantly ($P < 0.05$) decreased the soluble protein, SOD, POD, CAT activities while increased the TBARS and nitrite concentration in testis tissue homogenate. Catalase and superoxide dismutase levels were reestablished ($P < 0.05$) by giving *P. chinensis* ethanolic bark fraction. Co-administration of PCBE with CCl_4 significantly ($P < 0.05$) decreased the raised concentration of lipid peroxidation and nitrite content also seemed to be near to normal level.

3.2.2. Effect of PCBE extract on activity of GSH, GST, QR, GSH-Px and GSR in rat testis

Inhibitory effects of PCBE against CCl_4 toxicity on GSH and glutathione enzymes like, GSR, GST, GP-x of testis is displayed in Table 2. Prolonged administration of CCl_4 for about four weeks significantly ($P < 0.05$) reduced the activities of GST, GSH, GSR, GPx and QR in tissues of testes as compared to control group. Later on by administering PCBE extract reinstated the glutathione level GST, GSR, GPx, GSH and QR at various points. CCl_4 intoxicated rats when administered with silymarin it improved the damages of CCl_4 and inverted the enzymatic level to the control group. Though, groups given low and high concentration of PCBE and alone revealed nontoxic dose level, and those parameters were close to control group.

3.2.3. Effects of PCBE on testis protein, TBARS and nitrite activity in rat testis

The protective effect of PCBE on the level of testis protein, TBARS and nitrite content is presented in Table 2. The level TBARS and nitrite broadly ($P < 0.05$) increased in testis of rats after CCl_4 treatment while level of proteins decreased. Administration of PCBE along with CCl_4 considerably ($P < 0.05$) decreased the level of TBARS and nitrite while increased tissue protein level, reversing the intoxication effects of CCl_4.

3.2.4. Effects of P. chinensis bark on male testosterone level

Potential biomarkers of testicular toxicity are reproductive hormones. CCl_4 intoxication results in the secretion of numerous endocrine and pituitary glands. The protective effect of PCBE extract against CCl_4 intoxication on the serum testosterone level is shown in Table 2. Oral administration of PCBE extract reverted back significantly ($P < 0.05$) the testosterone level. Silymarin treatment removed CCl_4 intoxication and reduced the level of testosterone in serum of rat. Though, by administration of PCBE alone, serum level of hormones was close to control group. The overall progression of low and high concentration of PCBE against hormonal level of testis was found to be more effective at high concentration.

3.2.5. Histology

Present study aimed to evaluate the free radical scavenging activity of PCBE against CCl_4 that causes alterations in male reproductive system involving changes in antioxidant enzymes levels, hormones and histology. Microscopic parts derived from the control group male reproductive system revealed many germ

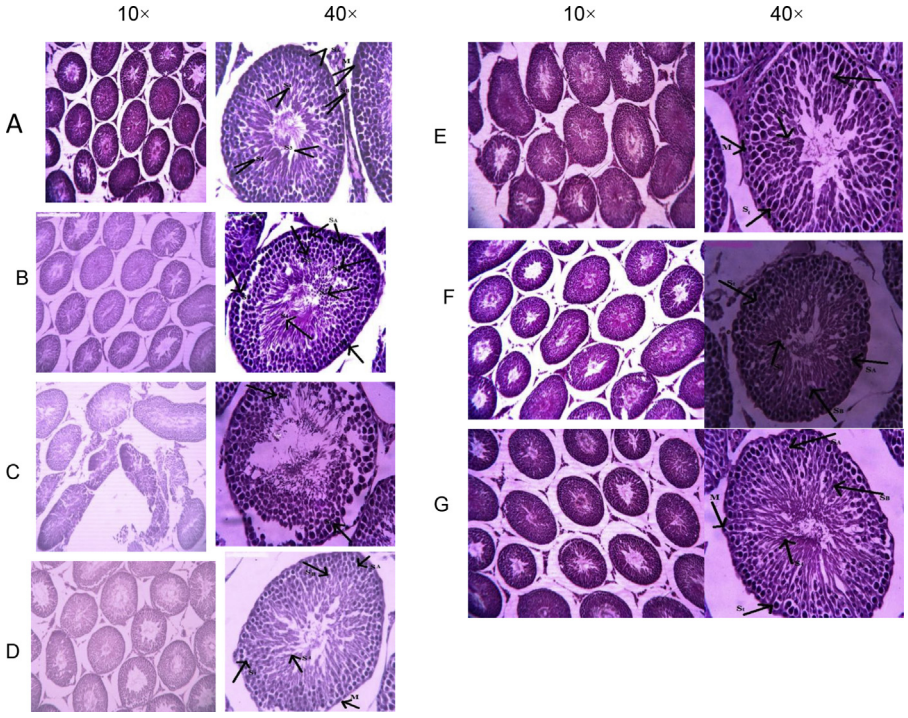

Figure 1. Testicular microphotographs of rat (H & E stain) at 10× and 40× magnification.
(A) histology section of testis from the control group showing normal histology with SA, type A spermatogonia, SB, type B spermatogonia, S1 primary spermatocytes, S3 spermatid, S4 pointed spermatozoa and M myofibroblast; (B) DMSO + Olive oil; (C) CCl_4; (D) PCBE + silymarin; (E) PCBE low dose + CCl_4; (F) PCBE high dose + CCl_4; (G) PCBE alone. PCBE: *P. chinensis* bark ethanol extract.

cells in the seminiferous tubules as shown in Figure 1A. Sertoli cells were also remarkable. In the middle of tubules small dark colored and oblong shaped sperms are seen. Glands having tall columnar epithelial cell lining are well differentiated. These cells lack prominent nucleoli. Microscopic sections of male reproductive system showed all the epithelial cells in the normal arrangement. Sperms with normal concentration and morphology were seen in the seminiferous tubules. The CCl_4 intoxication caused ruthless damages in testes with vacuolization of germinative epithelium, meiotic interruption, marked decrease in germ cells, and interstitial cells were disordered from seminiferous tubules and basement membrane. Seminiferous tubules of almost all the animals were abnormal, degeneration was also observed in few and Sertoli cells were present only. Sperms with broken and abnormal morphology were visible as shown in Figure 1C. Microscopic sections of the male reproductive system of Silymarin and CCl_4 group showed histoarchitecture near to control group because silymarin produced protective effect against CCl_4 injuries in Figure 1D.

Groups administered with PCBE revealed increased sperm concentration and maintenance and organization of seminiferous tubules. The protective effect of PCBE high dose was more against testicular damage Figure 1E. Normal tissue structure of testis was apparent in groups that were treated with high dose of PCBE. The results achieved from the histological study showed constancy with antioxidant status and hormonal tests.

4. Discussion

Medicinal plants are used to treat various ailments because the bioactive compounds present in this act as natural antioxidants, reducing agents or free radical scavengers. With the aid of free radical scavenging activity of numerous plant components, medicinal plant and herbs have gained much attention in research because they have less side effects, ease of availability and economically persistent as compared to synthetic drugs. Free radicals are involved in the generation of many ailments so medicinal plants are potent sources to scavenge these reactive species and prevent its chain reaction. Therefore, medicinal plant products are widely used to treat cancer, arteriosclerosis, cardiovascular, diabetes, aging, hepatitis and much more [22–24].

Medicinal plants have much influence on living systems due to their antioxidative therapeutic potential. The antioxidant activity could be determined by many *in vitro* assays so in the present study nine *in vitro* antioxidant assays were performed to measure the antioxidant behavior of *P. chinensis* plant extracts prepared in two different solvent system methanol and ethanol. To certify the presence of natural antioxidant different model assays were performed. The present study data displayed that plant extracts PCLE and PCBE showed best results as DPPH radical scavenger and it was due to the bioactive components which were extracted in different solvents. However, the bark of the respective plant in both solvents also showed good results. DPPH is a nitrogen-centered stable radical which by donation of electron or hydrogen gets reduced and color changes from violet to yellow. Substances carrying out this reaction are termed as antioxidants or free radical scavengers [25]. Extracts with higher flavonoid and phenolic contents usually have best DPPH scavenging property [26].

Hydrogen peroxide is not much reactive itself but may provide the hydroxyl radical that is toxic to cells. Therefore scavenging of hydrogen peroxide is very necessary for cell antioxidant defense [27]. In the present study among all extracts, PCLE showed the highest activity against hydrogen peroxide while other extracts PCBE, PCLM, and PCBM also have significant potential. Phenolic content of the plant extract may be attributed to causing the scavenging of H_2O_2 by electron donation and reducing H_2O_2 to water [28]. Thus the antioxidant compounds of *P. chinensis* plant extracts may be involved in the hydrogen peroxide radical scavenging.

ABTS radical scavenging activity is used to assess antioxidant potential of plant extracts and it involves the chemically produced drastic radical involves drastic radical [29]. The effect of the *P. chinensis* plant extracts prepared in methanol and ethanol solvent system showed ABTS radical scavenging with PCBE extract having a notable propensity against these radicals.

Beta-carotene bleaching assay is mostly done to calculate the antioxidant capacity of plant extract. In beta-carotene bleaching assay the yellow color of β-carotene is lost due to its interaction with radicals generated in the emulsion as a result of linoleic acid oxidation. The b-carotene bleaching rate can be decelerated by antioxidants present in the sample [30]. The data explained that PCBE extract showed highest beta-carotene bleaching activity and it was also significantly higher than standard. Other extracts PCBE, PCLM, and PCBM also have the notable activity to scavenge the free radicals.

Superoxide radical is notorious species as it acts as a precursor of other reactive species in cellular environment [31]. The present study revealed that PCBE showed markedly scavenging behavior against superoxide radical and hampered its propagation.

The hydroxyl radical is very reactive species among oxygen radicals and generates damages to adjacent biomolecules like DNA, protein and induces lipid peroxidation in lipids [32]. Hydroxyl radical (OH^-) is also involved in inflammatory disorders like arthritis [33]. Present study results suggested PCBE showed better scavenging activity than PCLM, PCBM, and PCLE respectively. Thus the polyphenolic compounds in these extracts may be liable for termination of chain reaction involved in lipid peroxidation.

An increase in the lipid peroxidation causes oxidative stress and changes the levels of enzymatic and non-enzymatic antioxidants. It is reported that many enzymatic and non-enzymatic processes had opted in a living system for the survival of free radicals and ROS. Methanol extracts of PCBM and PCBE noticeably reduced lipid peroxidation as compared to ethanol extracts of leaves and bark. Previous studies also reported that Mentha extract stops the lipids peroxidation propagation in the complex lipid matrix of a biological membrane [34–36].

Nitric oxide is produced in the mammalian cells as a free radical and regulates many physiological processes. However nitric oxide production in excess leads to several diseases [37]. The property of the plant extracts to hamper the generation of nitric oxide is evaluated by generating the free radical environment by sodium nitroprusside. Data of the present study suggested that PCBM revealed the best nitric oxide scavenging activity and also significantly higher than standard chemical. Other extracts PCLM, PCBE, and PCLE also showed good antioxidant behavior. The work of Ialenti, Moncada [37] also supported the obtained results. Beneficial effects of nitric oxide inhibitors have been reported to inflammation and tissue damage injuries [33].

In vitro antioxidant assays evaluating the antioxidant potential of various extracts of *P. chinensis* plant were the most

important part of the present study because these confirmed the free radical scavenging property and it might be due to the presence of phenolics and flavonoids in satisfactory amount. As it is supported by many studies explained above. Thus present study concluded that *P. chinensis* extract could be a good source of health promoting natural antioxidants in the synthesis natural drugs against various diseases. It was further confirmed by studying the *in vivo* effects of *P. chinensis* ethanol bark extract against chemically induced oxidative stress and antioxidant capacity was evaluated.

CCl_4 provoked poisonousness in male rats, displays that PCBE presumes an imperative part as a self-protective constituent for CCl_4 provoked harmfulness. Many chemicals including diverse environmental toxic agents and even clinically functional medications can result in intense cellular injuries in distinctive organs [38]. CCl_4 facilitates its testicular toxic properties and was explained [39] who described that chronic availability of CCl_4 recommends cellular autophagy with potential development of testicular damage induced by CCl_4 and augmented catabolic tissue. Current research shows that CCl_4 encouraged a valuable reduction in CAT, POD and SOD profile, beat the GSH, γ-GT, GST level and raised lipid peroxidation (TBARS), and nitrite in testes. It has been stated that against reactive oxygen species various assay CAT, POD, SOD establish a group of guard [40]. Due to little action of antioxidant enzymes in the testes and reduced GSH, γ-GT level, the CCl_4-instigated oxidative stress which goes to the extreme; the innate antioxidant system flops to defend the organ. Increased lipid peroxidation or inactivation of the antioxidant enzyme system might be the reason for reduced activity of SOD in testes in CCl_4 treated model of the animal [41] investigated the effect of CCl_4 on SOD, CAT and lipid peroxidation.

CCl_4 provoked testes damages linked with high nitrite generation. Nitric oxide is lipid and water soluble free radical prepared from L-arginine in the vascular endothelium by the action of NO^- synthase catalysts. There might be two causes behind the elevation of tissue NO^- levels which occur after introduction to CCl_4 usage: initiation of neutrophils in damaged testicular tissue, increased synthesis in view of the injury of the vascular endothelium, or the initiation of neutrophils in injured testicular tissue, stimulating synthesis of NO^- [42]. In acidic pH nitrites can be converted into nitric oxide. Superoxide and nitric oxide anion have been responding to create peroxynitrite anions. These peroxynitrite anions react with biomolecules and oxidized it, which finally prompts lipid peroxidation [42]. Independent testicular spin of rats conveyed a large quantity of nitrite substance in the tissues of the testis [43]. Oral administration of PCBE reduced the level of nitrite in this work. Against CCl_4 destructiveness, free radical scavenging ability of PCBE plays a protective role. Spermatogenesis is a complex and advanced process [44]. Due to the presentation with dangerous chemicals, spermatogonial degeneration can come [45].

Earlier studies on histomorphology of testes showed shrinkage of the tubular thickness and in germinal epithelium, degenerative changes occurred because of testicular atrophy [46] after the presentation of poisonous materials. Similar toxic influences were likewise accounted in CCl_4 treated groups. The CCl_4 presentation produced testicular damage and collapse in histological architecture [47] who stated vacuolated spermatogonial cells, injured columnar epithelial layer, edematous enlarged Leydig cells, and alterations in the seminiferous tubules.

Abraham, Wilferd [38] indicated that oxidative injury to testicular proteins in rats caused by CCl4. Oxidative stress caused damage to the proteins is significant as it can play role in hampering the DNA repair enzymes and secondary damages resulting in loss of reliability of damage polymerases during DNA replication [48] reported that DNA damage in various tissues like testis, brain, and liver. From the current study, it can be predictable that CCl_4 chronic exposure to the cells causes buildup of many toxic species, therefore, injuring both lipids and DNA. Indeed, by treating the several fractions of different plant extracts nullify the deteriorating effects on DNA. Recent studies clearly suggest the protective action of several samples against oxidative stress caused by CCl_4 and delivers verification about its medicinal use in reproductive disorders.

In the current study, CCl_4 treatment reduced the serum level of testosterone in rats shows either direct effects of chemical (CCl_4) at Leydig cell level or indirect effects by disturbing the hormonal environment at hypothalamic-pituitary axis [49] due to oxidative trauma in CCl_4 treated rats. In the current study, testosterone concentration was counted and was detected altogether high in association to that of the standard control groups. Treatment of animals with CCl_4 with PCBE upgrade the harmful influences of CCl_4 and the level of testosterone was enlarged, in a dose-dependent way. Administration of PCBE alone to the rat, slightly improved the serum level of testosterone. In addition to scavenging action of free radicals, this fraction would directly energize the specific key nervous process.

In vivo study revealed that CCl_4 induced oxidative stress could be released through PCBE extract of the plant because it significantly recovered the decreased levels of antioxidant enzymes approximately to control level. Serological and histological studies further showed the ameliorated effect and confirmed organs normalcy.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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