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Full Length Research Paper

Evaluation of antioxidant and fertility effects of *Digera muricata* in male rats

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Treatment of carbon tetrachloride (CCl_4) 1 ml/kg b.w. (10% in olive oil) to Sprague-Dawley male rats once a week for 16 weeks caused a significant increase in serum level of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), cholesterol, low density lipoprotein (LDL), direct bilirubin, total bilirubin, estradiol and prolactin; whereas suppressed level of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were restored with the administration of n-hexane extract of *Digera muricata* (DMH). In addition, hepatic activities of antioxidant enzymes; catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GSH-Px), glutathione reductase (GSR), quinone reductase (QR), gamma glutamyltransferase (γ-GT) suppressed with CCl_4 were elevated with DMH gavages. Decrease in hepatic contents of glutathione (GSH) while increase in thiobarbituric acid reactive substances (TBARS), nitrite and H_2O_2 contents with CCl_4 were returned towards the normal level with DMH. Treatment of DMH attenuates the toxicity of CCl_4 and the decrease in weight of body, liver, testis, accessory organs reversed towards the normal level. Presence of bioactive constituents (rutin and hyperoside) in DMH suggested to have therapeutic effects against CCl_4 induced oxidative stress and hypogonadism.

Key words: *Digera muricata*, antioxidant, thiobarbituric acid reactive substances, liver cirrhosis, carbon tetrachloride, testosterone.

INTRODUCTION

Liver cirrhosis is a worldwide health problem. Cirrhosis is a complication of many liver diseases that is characterized by abnormal structure and function of the liver. Hypogonadism (characterized by low testosterone levels and relative hyperestrogenism, loss of libido, sexual impotence and feminine body features in men) is a common complication of advanced liver cirrhosis (Van Thiel, 1981). There are reports of a prevalence of hypogonadism in 70 to 80% of cirrhotic patients (Fagiuoli et al., 1995). The production of testosterone by cirrhotic individuals is, on average, 25% of that found in normal individuals (Gelding et al., 1992).

Carbon tetrachloride is used extensively in experimental models to induce oxidative stress in rats (Handa and Anupama, 1990; Onori et al., 2000; Nabeshima et al., 2006). A single dose of CCl_4 can rapidly lead to both oxidative stress and acute liver injuries such as centrilobular necrosis and steatosis in rats (Weber et al., 2003; Lin et al., 2008). Liver injuries induced by CCl_4 are mediated through the formation of reactive intermediates such as trichloromethyl ($^{\bullet}CCl_3$) free radicals and ROS (Lin et al., 2008). Experimental and clinical results indicate that oxidative stress may be the link connecting different types of chronic liver injuries and hepatic fibrosis (Lin et al., 2008). The intracellular concentration of ROS is a consequence of both their production and their removal by various antioxidants. Thus, the antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4-induced hepatopathy (Weber et al., 2003; Yuan et al., 2008).

The reactive oxygen species from CCl_4 induces rat liver cirrhosis that resembles the human disease, and it can serve as a suitable animal model for studying human liver cirrhosis. Considering the hazards of treatment failure, drug resistance and heavy costs associated with current hepatic therapy, medicinal plants have attracted interest of many researchers in this field (Lakho and Rohra, 2006).

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Previous data demonstrated that rats with advanced cirrhosis caused by CCl_4 showed reduced testicular size and severe histopathological testicular abnormalities and loss of the germinal line (Khan and Ahmed, 2009).

Digera muricata is a common weed found during the summer season throughout the plains of Pakistan. *D. muricata* ethnopharmacologically has been used in renal disorders (Anjaria et al., 2002) aperient, refrigerant (Hocking, 1962). Antioxidant properties of *D. muricata* against the CCl_4-induced toxicity for kidneys (Khan et al., 2009) and testis had been documented (Khan and Ahmed, 2009). This plant is used as an alternative for secondary infertility (Chettleborough et al., 2000). Secondary infertility is found to be associated with hepatic disorders (Gelding et al., 1992). The models created by the use of CCl_4 to induce liver injuries can be best suited to study the hypogonadism in rat. To check this hypothesis, this study was designed to evaluate the protective effects of the n-hexane extract of *D. muricata* on hypogonadism in cirrhotic rats. In this study aside from the determination of CCl_4 induced toxicity on liver, testis and accessory organs, n-hexane extract of *D. muricata* (DMH) was also characterized for the presence of various bioactive constituents.

MATERIALS AND METHODS

Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, gamma-glutamyl p-nitroanilide, glycylglycine, bovine serum albumin (BSA), 1,2-dithio-bis nitro benzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), CCl_4, flavine adenine dinucleotide (FAD), glucose-6-phosphate, Tween-20, 2,6-dichlorophenolindophenol, thiobarbituric acid (TBA), picric acid, sodium tungstate, sodium hydroxide, trichloroacetic acid (TCA) and perchloric acid (PCA) of Sigma Chemicals Co. USA were purchased from the local market.

Preparation of n-hexane extract of *D. muricata* (DMH)

Mature plants of *D. muricata* L. (Mart.) locally named as “Tandla” or “Lulur” were collected and a voucher specimen #125127 has been deposited in the herbarium of Pakistan. The authenticated and freshly collected aerial parts of the plant were chopped, shade dried and 750 g powder was exhaustively extracted with n-hexane at room temperature. The solvent was removed under reduced pressure to obtain a viscous yellowish green 4.5 g of (0.6% of initial amount) extract (DMH).

Phytochemical studies

Flavonoids in DMH were quantitatively determined (in triplicate) according to Boham and Kocipai (1974), alkaloids (Harborne, 1973), tannins (Van-Buren and Robinson, 1981) and saponins (Obadoni and Ochuko, 2001). Qualitative studies of DMH for the presence of flavonoids, alkaloids, terpenoids and saponins were carried out according to Harborne (1973), tannins (Sofowara, 1993) while coumarins, cardiac glycosides , anthraquinones and phlobatannins by Trease and Evans (1989).

Determination of total phenolic contents

The amount of total phenolics in extracts was determined according to the Folin-Ciocalteu procedure (Singleton et al., 1999). Samples (200 µl, three replicates) were introduced into test tubes; 1.0 ml of Folin-Ciocalteu’s reagent and 0.8 ml of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry weight of DMH. Experiment was performed in triplicate.

Determination of total flavonoid contents

Flavonoids (extracted with 5% $NaNO_2$, 10% $AlCl_3$ $6H_2O$ and 1 M NaOH) were measured at 510 nm with a known rutin concentration as a standard (in triplicate). The results were expressed as milligrams of rutin equivalents (RE) per gram dry weighty of DMH (Singleton et al., 1999).

High performance liquid chromatograhy (HPLC) of DMH

Sample preparation

50 mg of MCL was extracted with 6 ml of 25% hydrochloric acid and 20 ml methanol for 1 h. The obtained extract was filtered to a volumetric flask. The residue was heated twice with 20 ml of methanol for 20 min to obtain the extract. The combined extract was diluted with methanol to 100 ml. 5 ml portion of the solution was filtered and then was transferred to a volumetric flask and diluted with 10 ml of methanol. The sample (10 µl) was injected into the HPLC apparatus.

HPLC determination

Samples were analyzed on Agilent HPLC system. Separation was carried out through column 20RBAX ECLIPSE, XDB-C18, (5 µm; 4.6 x 150 mm, Agilent USA) with UV-VIS Spectra-Focus detector, injector-auto sampler. Solvent A (0.05% trifluoroacetic acid) and solvent B (0.038% trifluoroacetic acid in 83% acetonitrile (v/v) with the following gradient: 0 to 5 min, 15% B in A, 5 to 10 min, 50% B in A, 10 to 15 min, 70% B in A. The flow rate was 1 ml/min and injection volume was 10 µl. Eleven standard compounds including rutin, myricetin, vitexin, orientin, hyperoside, isovitexin, isoquercetin, luteolin, apigenin, kaempherol, and luteolin-7-glucoside were run for comparative detection and optimized. The calibration curves were defined for each compound in the range of sample quantity 0.02 to 0.5 µg. All samples were assayed in triplicate.

Animals and treatment

The experimental protocol and procedures used in this study were approved by the Ethics Committee of the Quaid-i-Azam University, Islamabad, Pakistan for the care and use of laboratory animals.This study was carried out on healthy male albino rats of three months old weighing 250 to 260 g. 70 rats were provided by the Animal House of National Institute of Health (NIH) Islamabad and were maintained at the Primate Facility of Quaid-i-Azam University, Islamabad. The animals were housed in conventional cages with free access to water and food at 21± 2°C with a 12 h light-dark cycle. Animals were divided into seven groups of ten rats each and

their protocol scheme is given as under (Table 1).

At the end of experimental period of 16 weeks, a midline abdominal incision was performed and blood samples were collected from atrium in routine biochemical test tubes having EDTA and centrifuged at 2000 x g for 10 min at 4°C to get the serum. Liver, male gonads along with accessory organs were removed immediately and weighted. A part of the liver was fixed while other was dried in liquid nitrogen and stored at -70°C for various biochemical and enzymatic analysis. Histopathological studies of liver, testis and epididymis was carried out of 4 to 5 µm thin sections.

Assessment of biochemical markers

Serum analysis of various liver marker enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphates (ALP), lactate dehydrogenase (LDH), total cholesterol (TCH), high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TG), direct bilirubin and total bilirubin were estimated by using standard AMP diagnostic kits (Stattogger Strasse 31b 8045 Graz, Austria).

Assessment of antioxidant enzymes

Liver tissue was homogenized in 10 volume of 100 mmol KH_2PO_4 buffer containing 1 mmol EDTA (pH 7.4) and centrifuged at 12,000 x g for 30 min at 4°C. Protein concentration of the supernatant of liver tissue was determined by the method of Lowry et al. (1951) using crystalline BSA as standard.

Catalase (CAT) and peroxidase (POD) activities were determined by the method of Chance and Maehly (1955) with some modifications. Briefly CAT activity was determined by adding H_2O_2 at 240 nm while POD activity was measured by using guaiacol as substrate at 470 nm. One unit of CAT and POD activity was defined as an absorbance change of 0.01 as units/min. Superoxide dismutase (SOD) activity was estimated by using phenazine methosulphate and sodium pyrophosphate buffer according to Kakkar et al. (1984). Enzyme reaction was initiated by adding NADH (780 µmol) and stopped after 1 min by adding glacial acetic acid and color intensity at 560 nm was recorded. Results are expressed in units/mg tissue protein.

Assessment of phase II enzymes

Induction of lipid peroxidation by CCl_4 and its protection by the DMH was determined by the estimation of various enzyme activities and thiobarbituric acid reactive substances (TBARS) contents.

Glutathione-S-transferase assay (GST)

Liver glutathione-*S*-transferase activity was determined according to Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. GST was measured at 340 nm using a molar extinction coefficient of 9.6 x $10^3 M^{-1}cm^{-1}$.

Glutathione reductase assay (GSR)

Glutathione reductase activity (Carlberg and Mannervik, 1975) was measured at 340 nm by using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate. An extinction coefficient of 6.22 x $10^3 M^{-1}cm^{-1}$ was used for calculation.

Glutathione peroxidase assay (GSH-Px)

Glutathione peroxidase (GSH-Px) activity was measured by using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate (Mohandas et al., 1984). An extinction coefficient of 6.22 x $10^3 M^{-1}cm^{-1}$ at 340 nm was used for calculation.

γ-glutamyl transpeptidase assay (γ-GT)

γ-GT activity was determined by the method of Orlowski and Meister (1973) using glutamyl *p*-nitroanilide as substrate and activity was determined at 405 nm. Enzyme activity was calculated as nmol *p*-nitroaniline formed/min/mg protein using a molar extinction coefficient of 1.74 x $10^3 M^{-1}cm^{-1}$.

Quinone reductase assay (QR)

The activity of quinone reductase was determined according to Benson et al. (1980). The reduction of dichlorophenolindophenol (DCPIP) was recorded at 600 nm and enzyme activity was calculated as nmol of DCPIP reduced/min/mg protein using molar extinction coefficient of 2.1 x $10^4 M^{-1}cm^{-1}$

Reduced glutathione assay (GSH)

Reduced glutathione was estimated by the method of Jollow et al. (1974) by using 1,2-dithio-bis nitro benzoic acid (DTNB) as substrate. The yellow color developed was read immediately at 412 nm and expressed as µmol GSH/g tissue.

Estimation of lipid peroxidation assay (TBARS)

Liver thiobarbituric acid-reactive substances (TBARS) were measured at 535 nm by using 2-thiobarbituric acid (2,6-dihydroxypyrimidine-2-thiol; TBA). An extinction coefficient of 156,000 $M^{-1} cm^{-1}$ was used for calculation according to Wright et al. (1981) as modified by Iqbal et al. (1996).

Hydrogen peroxide assay (H_2O_2)

Hydrogen peroxide (H_2O_2) was assayed by using H_2O_2-mediated horseradish peroxidase-dependent oxidation of phenol red by the method of Pick and Keisari (1981) based on the standard curve of H_2O_2 oxidized phenol red.

Nitrite assay

Supernatant of homogenate was collected after deproteinized with NaOH and $ZnSO_4$ and centrifuged at 6400 x g for 20 min. Griess reagent was used to blank the spectrophotometer at 540 nm and supernatant was added. Nitrite concentration was calculated using a standard curve for sodium nitrite.

Histopathalogical studies

For microscopic evaluation tissues of liver, testis and epididymis were fixed in a fixative (absolute ethanol 60%, formaldehyde 30%, glacial acetic acid 10%) and embedded in paraffin, sectioned at 4 µm and subsequently stained with hematoxylin/eosin. Sections were studied under light microscope (DIALUX 20 EB) at 40 and 100 magnifications.

Table 1. Animals divided into seven groups and their protocol scheme.

Treatments	Groups						
	I	II	III	IV	V	VI	VII
Olive oil (1 ml/kg body weight i.p.)	–	+	+	+	+	+	+
DMSO (1 ml/kg body weight orally)	–	+	+	+	+	+	+
CCl_4 (1 ml/kg body weight i.p., 10% in olive oil)	–	–	–	+	+	+	+
DMH (100 mg/kg body weight orally)	–	–	–	–	+	–	–
DMH (150 mg/kg body weight orally)	–	–	–	–	–	+	–
DMH (200 mg/kg body weight orally)	–	–	+	–	–	–	+

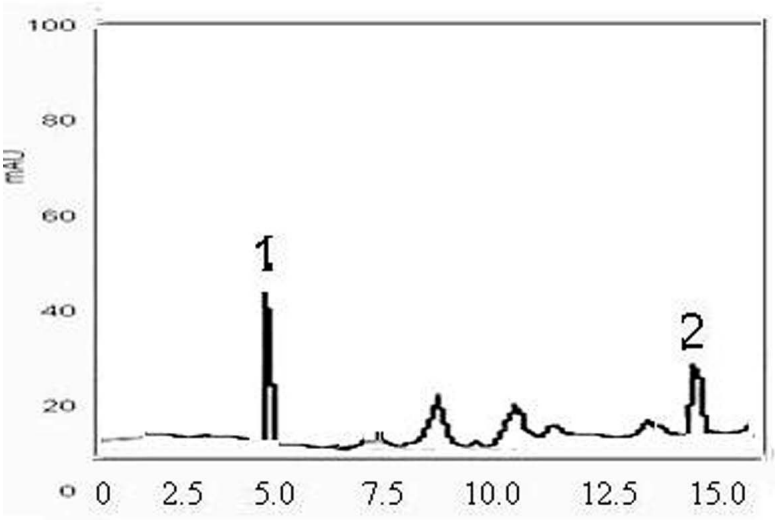

Figure 1. HPLC studies of DMH. (1) hyperoside and (2) rutin.

Assessment of fertility hormones

Serum level of testosterone (Ref. 6C28), luteinizing hormone (LH) (Ref. 6C25), follicle stimulating hormone (FSH) (Ref. 6C24), estradiol (Ref. 2K25) and prolactin (Ref. B7K760) was estimated on a fully automated by Abbott ARCHITECT®. Standards, samples and controls were analyzed in duplicate in a single batch. In all assays, the coefficient of intra- and inter-assay variability was below 10%.

Statistical analysis

All the group data were statistically evaluated with SPSS 13.0 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean ± SD for ten animals in each group.

RESULTS

Biochemical composition of DMH

Analysis of DMH confirmed the presence of flavonoids, alkaloids, terpenoids, saponins, tannins and cardiac glycosides while anthraquinones, coumarins and phlobatanins were absent.

Quantitative analysis of DMH

Percentage of dry weight basis for flavonoids which is (2.81±0.28), saponins (1.55±1.18), alkaloids (0.35±0.026) and tannins (0.20±0.028) had been determined in DMH.

Total phenolic and flavonoid contents

Total phenolic contents as equivalent to gallic acid were 52±1.02 mg/g of the extract while the total flavonoid contents were 15.02±1.2 mg/g extract as equivalent to rutin in DMH.

HPLC studies of DMH

Accordingly, hyperoside, and rutin were found; the known flavonoids in DMH by HPLC studies. Hyperoside (2.8 µg/mg of DMH) and rutin (1.02 µg/mg of DMH) were recorded in DMH (Figure 1).

Table 2. Effect of DMH on liver marker enzymes in rat.

Treatment	ALT (U/l)	AST (U/l)	ALP (U/l)	LDH (U/l)
Control	49.0±5.1b	84.2±4.5b	202.3±15.0b	45.0±5.1b
Olive oil (1 ml/kg)+DMSO (1 ml/kg)	50.1±4.1b	85.7±6.0b	196.5±12.5b	45.0±3.8b
DMH (200 mg/kg)	47.2±4.9b	82.1±5.7b	187.2±21.3b	44.0±4.5b
CCl_4 (1 ml/kg, 10% in olive oil)	97.3±7.5A	216.5±8.5A	394.1±18.3A	94.9±7.7A
CCl_4 (1 ml/kg)+ DMH (100 mg/kg)	93.7±6.0A	204.2±10.4A	380.1±27.0A	90.4±5.9A
CCl_4 (1 ml/kg)+ DMH (150 mg/kg)	76.4±5.3Ab	157.0±8.8Ab	302.2±14.3Ab	72.3±5.5Ab
CCl_4 (1 ml/kg)+ DMH (200 mg/kg)	66.3±5.3Ab	148.2±9.5Ab	260.6±15.4Ab	59.2±5.7Ab

Mean±SD (n=10), A=P<0.001significance from control group. a=P<0.05; b=P<0.001significance from CCl_4 group.

Table 3. Effect of DMH on serum level of biochemicals in rat.

Treatment	Triglycerids (U/l)	Total cholesterol (U/l)	HDL (U/l)	LDL (U/l)	Direct bilirubin (mg/dl)	Total bilirubin (mg/dl)
Control	8.0±0.8b	6.3±0.7b	3.52±0.4b	2.8±0.3b	1.09±0.12b	1.62±0.18b
Olive oil (1 ml/kg)+DMSO (1 ml/kg)	8.2±0.6b	6.5±0.6b	3.64±0.3b	2.9±0.2b	1.07±0.08b	1.64±0.12b
DMH (200 mg/kg)	7.9±0.8b	6.0±0.7b	3.54±0.3b	2.7±0.3b	1.07±0.09b	1.55±0.17b
CCl_4 (1 ml/kg, 10% in olive oil)	16.4±1.3B	13.1±1.0B	2.57±0.2B	10.6±0.8B	1.68±0.13B	2.28±0.12B
CCl_4 (1 ml/kg)+ DMH (100 mg/kg)	15.5±1.2Ba	12.6±0.8B	2.87±0.2Ba	9.7±0.6Ba	1.62±0.11B	2.22±0.11B
CCl_4 (1 ml/kg)+ DMH (150 mg/kg)	12.7±0.9Bb	10.2±0.7Bb	3.00±0.2Bb	7.2±0.5Bb	1.48±0.10Bb	2.08±0.10Bb
CCl_4 (1 ml/kg)+ DMH (200 mg/kg)	10.8±1.0Bb	8.8±0.7Bb	3.12±0.1Ab	5.8±0.5Bb	1.15±0.09b	1.75±0.09b

Mean±SD (n=10), A=P<0.01; B=P<0.001significance from control group.a=P<0.05; b=P<0.001significance from CCl_4 group.

Effect of DMH on liver marker enzymes

The effect of CCl_4, DMH and CCl_4+DMH was determined for the activities of liver marker enzymes. Serum level of hepatic enzymes; ALT, AST, ALP and LDH was significantly elevated in the CCl_4 treated rats. DMH consumption appeared to lower the severity of the CCl_4 treatment (Table 2). The activities of ALT, AST, ALP and LDH were significantly increased, dose-dependently, in CCl_4+DMH groups as compared to the CCl_4 treated rats. The activities of ALT, AST, ALP and LDH were remained statistically similar (P>0.05) to control group with the treatment of DMH (200 mg/kg body weight).

Effect of DMH on serum biochemicals

The serum levels of hepatic biochemical markers, such as direct and total bilirubin, TCH, TG, LDL were significantly elevated in the CCl_4-treated animals (Table 3). However, serum level of total protein, albumin and HDL was reduced in the CCl_4 treated rats. Administration of DMH during CCl_4 treatment significantly lowered, in a dose-dependent manner, the serum direct and total bilirubin, TCH, TG, LDL as compared with those of CCl_4 treated group. However, total protein, albumin was elevated with DMH in a concentration dependent manner. Treatment of DMH (200 mg/kg body weight) alone did not (P>0.05) change the level of these biomarkers as compared to the control group.

Effect of DMH on antioxidant enzymes

CCl_4 induced hepatotoxicity can also be determined by recording the perturbations of antioxidant defense system (Table 4). CCl_4 caused significant decrease in the activities of CAT, POD and SOD enzyme. In contrast, the activity of CAT, POD and SOD was profoundly increased, in a dose dependent way, with DMH consuming CCl_4 treated rats. CCl_4 induced toxicity to antioxidant enzymes was also associated with the elevation of thiobarbituric acid reactive substances (TBARS) of hepatic tissues. This was accompanied by decreased level of GSH, while enhanced level of nitrite and H_2O_2 contents in the liver (Table 5).

Taken together, these results indicate that CCl_4 elicited production of free radicals and suppression of antioxidant enzymes causing significant damage to hepatic tissues of rat. An addition of DMH at various doses in diet reversed the perturbation of these parameters towards the control level.

Effect of DMH on lipid peroxidation

The toxicity of CCl_4 significantly decreased the liver GST,

Table 4. Effect of DMH on antioxidant enzymes, nitrite and H_2O_2 contents of liver in rat.

Treatment	CAT (U/min)	POD (U/min)	SOD (U/mg protein)	Nitrite (µmol/ml)	H_2O_2 (nmol/mg tissue)	TBARS (nmol/mg protein)	GSH (nmol/g tissue)
Control	5.0±0.6c	10.6±1.2c	20.0±2.5c	55.1±6.2c	2.0±0.2c	30.3±3.4b	1.60±0.2b
Olive oil (1 ml/kg)+DMSO (1 ml/kg)	5.1±0.5c	10.8±1.0c	20.7±2.0c	56.5±5.0c	2.1±0.2c	31.0±2.7b	1.57±0.1b
DMH (200 mg/kg)	4.8±0.5c	10.1±1.1c	19.4±1.9c	52.9±6.0c	2.0±0.2c	29.1±3.3b	1.69±0.1b
CCl_4 (1 ml/kg, 10% in olive oil)	2.8±0.2B	7.7±0.6B	11.4±1.2B	105.2±8.4B	4.3±0.3B	54.4±4.4A	1.11±0.8A
CCl_4 (1 ml/kg)+ DMH (100 mg/kg)	3.1±0.2B	8.6±0.6Ba	12.5±1.4B	94.1±6.4Bc	4.2±0.4B	53.7±4.8A	1.19±0.1A
CCl_4 (1 ml/kg)+ DMH (150 mg/kg)	3.5±0.3Bc	9.0±0.7Bb	14.4±1.0Bc	73.7±5.4Bc	3.1±0.2Bc	39.6±2.9Ab	1.34±0.1Ab
CCl_4 (1 ml/kg)+ DMH (200 mg/kg)	4.1±0.3Bc	9.2±0.7Ac	16.5±1.3Bc	66.5±5.7Bc	2.4±0.2Ac	31.6±2.7b	1.37±0.1Ab

Mean±SD (n=10), A=P<0.01; B=P<0.001significance from control group.a=P<0.05; b=P<0.01; c=P<0.001significance from CCl_4 group.

Table 5. Effect of DMH on TBARS, GSH and lipid peroxidation enzymes of liver in rat.

Treatment	GST (nmol/mg protein)	GSH-Px (nmol/mg protein)	GSR (nmol/mg protein)	QR (nmol/mg protein)	γ-GT (nmol/mg protein)
Control	196.5±17.2b	93.2±9.4b	126.8±14.4b	120.2±13.6b	98.1±7.1b
Olive oil (1 ml/kg)+DMSO (1 ml/kg)	198.2±11.7b	94.6±9.3b	127.2±9.4b	120.1±9.9b	100.2±7.7b
DMH (200 mg/kg)	200.5±9.6b	90.2±11.8b	121.7±13.8b	115.4±13.1b	99.4±9.2b
CCl_4 (1 ml/kg, 10% in olive oil)	114.0±8.5A	45.4±3.6A	65.8±5.3A	68.0±5.5A	196.0±13.6A
CCl_4 (1 ml/kg)+ DMH (100 mg/kg)	121.4±9.0A	49.9±3.4A	72.4±4.9A	74.9±5.0A	186.3±13.4Aa
CCl_4 (1 ml/kg)+ DMH (150 mg/kg)	141.2±10.6Ab	56.5±4.1Aa	120.2±13.6Ab	84.7±6.2Ab	152.8±10.6Ab
CCl_4 (1 ml/kg)+ DMH (200 mg/kg)	163.1±13.2Ab	65.5±5.6Ab	120.0±9.9Ab	98.3±8.4Ab	132.4±12.2Ab

Mean±SD (n=10), A=P<0.001significance from control group.a=P<0.01; b=P<0.001significance from CCl_4 group.

GSH-Px, GSR and QR levels while and γ-GT level increased that of the control group (Table 6). An addition of DMH at doses of 100, 150 and 200 mg/kg body weight in diet reversed the GST, GSH-Px, GSR and QR level in liver of rats. Especially high dose of DMH (200 mg/kg body weight) markedly restored the activities of these enzymes to the normal level.

Effect of DMH on serum level of hormones

CCl_4 administration for 16 weeks significantly decreased the serum level of testosterone, FSH, LH, while elevated the level of estradiol and prolactin against the control rats (Table 7). Treatment of various doses of DMH during CCl_4 treatment significantly enhanced, in a dose-dependent manner, the serum level of testosterone, FSH, LH, while lowered the level of estradiol and prolactin as compared with those of CCl_4 treated group. Especially high dose of DMH (200 mg/kg body weight) restored almost completely the prolactin to the normal level.

Effect of DMH on testis weight and accessory organs

The CCl_4 toxicity significantly decreased the testis weight and accessory organs that is cauput epididymis, cauda epididymis, seminal vesicle, ventral prostate along with decrease in diameter of seminiferous tubules. Drastic decrease in the weight of these organs indicated that excessive damage was induced with CCl_4 treatment. However, oral addition of DMH in rats treated with CCl_4, was able to reduce the CCl_4-induced changes in the weight and diameter of the above parameters in a dose dependent way (Tables 8 and 9).

Testicular histopathology

Histopathology of testis of control, vehicle and DMH group showed no marked changes. Micro thin sections from these three groups indicated the normal cycle of spermatogenesis. Seminiferous tubules had well preserved Sertoli cells and well delineated tubular basement membrane (Figure 2). The interstitium between tubules and Leydig cells were also intact in these groups. However, in the CCl_4-treated group, differences were observed in histology of testis. Although the tubular basement membranes of seminiferous tubules were identified in some areas, tubules could exhibit focal or diffuse intermediate necrosis. Most of the germ cells were

Table 6. Effect of DMH on serum level of hormones in rat.

Treatment	Testosterone (µg/ml)	Estradiol (µg/ml)	Prolactin (µg/ml)	Luteinizing Hormone (µg/ml)	Follicle stimulating Hormone (µg/ml)
Control	3.17±0.18 c	13.05±0.46 c	22.78±0.88 c	1.77±0.23 c	20.23±1.34 c
Olive oil (1 ml/kg)+DMSO (1 ml/kg)	3.19±0.28 c	13.25±0.66 c	23.00±0.65 c	1.80±0.42 c	19.76±1.12 c
DMH (200 mg/kg)	3.32±0.28 c	13.33±0.51 c	22.34±0.76 c	1.81±0.23 c	21.16±1.28 c
CCl_4 (1 ml/kg, 10% in olive oil)	1.46±0.27 C	18.43±0.68 C	28.26±1.00 C	1.14±0.14 C	17.14±1.18 C
CCl_4 (1 ml/kg)+ DMH (100 mg/kg)	1.77±0.27 Cb	18.00±0.56 C	26.50±1.44 Cc	1.22±0.09 C	17.73±0.63 C
CCl_4 (1 ml/kg)+ DMH (150 mg/kg)	2.06±0.33 Cc	17.07±0.75 Cc	25.74±1.34 Cc	1.36±0.05 Cb	18.45±1.54 Ba
CCl_4 (1 ml/kg)+ DMH (200 mg/kg)	2.32±0.13 Cc	16.20±0.90 Cc	22.13±0.81 c	1.51±0.12 Ac	19.00±0.45 Ab

Mean±SD (n=10), A=P<0.05; B=P<0.01; C=P<0.001significance from control group. a=P<0.05; b=P<0.01; c=P<0.001significance from CCl_4 group.

Table 7. Effect of DMH on body weight, testis and liver in rat.

Treatment	Initial body weight (g)	Final body weight (g)	% increase in body weight	Absolute testis weight (g)	Relative testis weight (as %) body weight	Absolute liver weight (g)	Relative liver weight (as %) body weight
Control	266.5±9.0	373±9.5	40.1±6.0 b	2.021±6.7 b	0.54±0.017 b	11.1±0.8 b	2.98±0.17 b
Olive oil (1 ml/kg)+DMSO (1 ml/kg)	268.9±8.6	372±8.0	38.6±5.6 b	2.028±6.5 b	0.54±0.019 b	11.4±0.6	3.06±0.17 b
DMH (200 mg/kg)	267.4±10.8	374±8.5	40.0±5.7 b	2.063±5.7 b	0.55±0.021 b	11.0±0.6 b	2.95±0.19 b
CCl_4 (1 ml/kg, 10% in olive oil)	271.7±7.1	338±8.0	24.5±4.3 B	1.468±4.4 B	0.42±0.031 B	11.8±0.6 A	3.51±0.20 B
CCl_4 (1 ml/kg)+ DMH (100 mg/kg)	266.8±7.8	347±9.0	30.4±4.2 Ba	1.559±5.6 Ba	0.44±0.023 B	11.7±0.6 A	3.37±0.23 B
CCl_4 (1 ml/kg)+ DMH (150 mg/kg)	264.2±6.1	354±11.4	34.1±5.8 Ab	1.692±5.6 Bb	0.47±0.026 Bb	11.4±0.6	3.21±0.17 Aa
CCl_4 (1 ml/kg)+ DMH (200 mg/kg)	266.5±8.6	374±8.9	40.7±5.4 b	1.950±5.9 Bb	0.52±0.017 b	11.4±0.5	3.04±0.22 Ab

Mean±SD (n=10), A=P<0.05; B=P<0.001significance from control group. a=P<0.01; b=P<0.001significance from CCl_4 group.

Table 8. Effect of DMH on cauput epididymis, cauda epididymis and vas deferens in rat.

Treatment	Absolute cauput epididymis weight (mg)	Relative cauput epididymis weight (as %) body weight	Cauda epididymis weight (mg)	Relative cauda epididymis weight (as %) body weight	Vas deferens weight (mg)	Relative vas deferens weight (as %) body weight (mg)
Control	395.5±23.6 b	0.106±0.004 b	261.9±8.8 b	0.070±0.001 a	101.9±7.7 b	0.027±0.002 b
Olive oil (1 ml/kg)+DMSO (1 ml/kg)	396.2±13.5 b	0.106±0.005 b	261.5±7.6 b	0.070±0.002 a	102.4±7.7 b	0.025±0.007 b
DMH (200 mg/kg)	398.7±18.5 b	0.106±0.005 b	262.9±7.4 b	0.070±0.002 a	102.7±5.4 b	0.027±0.001 b
CCl_4 (1 ml/kg, 10% in olive oil)	313.2±6.07 B	0.092±0.002 B	225.4±7.3 B	0.066±0.003 A	58.9±6.0 B	0.017±0.001 B
CCl_4 (1 ml/kg)+ DMH (100 mg/kg)	331.0±8.45 Ba	0.095±0.004 B	235.1±6.5 Ba	0.067±0.001 A	61.0±3.9 B	0.017±0.000 B
CCl_4 (1 ml/kg)+ DMH (150 mg/kg)	343.9±5.92 Bb	0.097±0.002 Ba	237.2±4.3 Bb	0.067±0.001 A	68.9±3.8 Bb	0.019±0.001 B
CCl_4 (1 ml/kg)+ DMH (200 mg/kg)	368.0±9.84 Bb	0.098±0.004 Ba	249.1±7.6 Bb	0.066±0.002 A	84.7±6.1 Bb	0.022±0.002 Aa

Mean±SD (n=10), A=P<0.05; B=P<0.001significance from control group. a=P<0.01; b=P<0.001significance from CCl_4 group.

Table 9. Effect of DMH on seminal vesicle, ventral prostate and seminiferous tubules in rat.

Treatment	Absolute seminal vesicle weight (mg)	Relative seminal vesicle weight (as %) body weight	Absolute ventral prostate weight (mg)	Relative ventral prostate weight (as %) body weight	Seminiferous tubule diameter (µm)
Control	333.3±6.7 b	0.089±0.003 b	175.8±5.7 b	0.047±0.002	256.9±8.7 b
Olive oil (1 ml/kg)+DMSO (1 ml/kg)	330.6±9.6 b	0.088±0.002 b	175.7±6.8 b	0.047±0.001	256.1±6.2 b
DMH (200 mg/kg)	333.9±6.2 b	0.089±0.002 b	172.5±8.2 b	0.046±0.002	258.0±6.9 b
CCl_4 (1 ml/kg, 10% in olive oil)	156.5±7.0 B	0.046±0.002 B	148.4±27.2 B	0.043±0.008	208.6±7.8 B
CCl_4 (1 ml/kg)+ DMH (100 mg/kg)	183.4±15.2 Bb	0.052±0.005 Bb	147.9±4.4 B	0.042±0.001 A	220.1±7.0 Ba
CCl_4 (1 ml/kg)+ DMH (150 mg/kg)	245.7±12.4 Bb	0.069±0.004 Bb	163.1±6.8 Aa	0.046±0.002	231.0±8.5 Bb
CCl_4 (1 ml/kg)+ DMH (200 mg/kg)	277.6±9.0 Bb	0.074±0.002 Bb	166.6±6.4a	0.044±0.001	240.1±6.6 Ab

Mean±SD (n=10), A=P<0.05; B=P<0.001significance from control group. a=P<0.01; b=P<0.001significance from CCl_4 group.

degenerated, especially the ones involving highly differentiated germ cells. Most of the sperms were deformed. Partially the ground substance within the interstitium also disappeared and replaced by fibroblast and inflammatory cells in some areas of testis. Treatment of DMH ameliorated the toxic effects of the CCl_4, in a dose dependent manner (Figure 2). Especially in the group treated with high dose of DMH (200 mg/kg body weight) tubular basement was well delineated, germinal layers, Leydig and Sertolli cells were present.

Epididymis histopathology

Epididymis showed normal histology in control, vehicle as well as DMH treated group. CCl_4 treatment caused degeneration of the membranes and lumens of epididymis was devoid of sperms (Figure 3). However, feeding of DMH of rats retard the toxicity of CCl_4 and the epididymis histopathology reversed towards the control architecture in a concentration dependent manner. These effects were more pronounced especially at the high dose of DMH (200 mg/kg body weight).

DISCUSSION

In this study, we demonstrated hepatoprotective effects of DMH against oxidative stress induced by CCl_4 exposure in rats. CCl_4 treatment enhanced lipid peroxidation through trichloromethyl radical ($^{\bullet}CCl_3$) formation from the metabolic conversion of CCl_4 by cytochrome P450. As O_2 tension rises, a greater fraction of $^{\bullet}CCl_3$ present in the system reacts very rapidly with O_2 and many orders of magnitude more reactive free radical, peroxy trichloromethyl ($CCl_3OO\bullet$) has been generated from $^{\bullet}CCl_3$. This radical is more reactive and is capable of abstracting hydrogen from polyunsaturated fatty acids (PUFA) to initiate the process of lipid peroxidation (Yuan et al., 2008). Reactive oxygen species (ROS) play a major role in the development of a wide variety of oxidative diseases (Halliwell and Whiteman, 2004).

Free radical formation during the metabolism of CCl_4 by hepatic microsome, cause lipid peroxidation of the cellular membrane leading to the necrosis of hepatocytes. Rats treated with CCl_4 developed significant hepatic damage and oxidative e stress as evidenced by substantial increase in the serum activities of ALT, AST, ALP, LDH, total and direct bilirubin that are indicators of cellular leakage and loss of functional integrity of cell membrane in liver (Szymonik-Lesiuk et al. 2003; Lin et al., 2008; Yuan et al., 2008). Centrilobular necrosis and portal disruption were established with CCl_4 in this study (Figure 4). Phosphatases are important and critical enzymes in biological processes, they are responsible for detoxification, metabolism and biosynthesis of energetic macromolecules for different essential functions. Any interference in these enzymes leads to biochemical impairment and lesions of the tissue and cellular function (Khan et al., 2001). The reduction in the levels of these parameters toward the respective normal values by DMH at two doses (150 and 200 mg/kg body weight) is an indication of the stabilization of plasma membranes as well as repair of hepatic tissue damage caused by CCl_4. This indicates that the anti-lipid peroxidation and/or adaptive nature of the systems brought about by DMH acted against the damaging effects of free radicals produced by CCl_4. Serum level of total triglycerides (TG) was significantly enhanced in CCl_4 treated group a

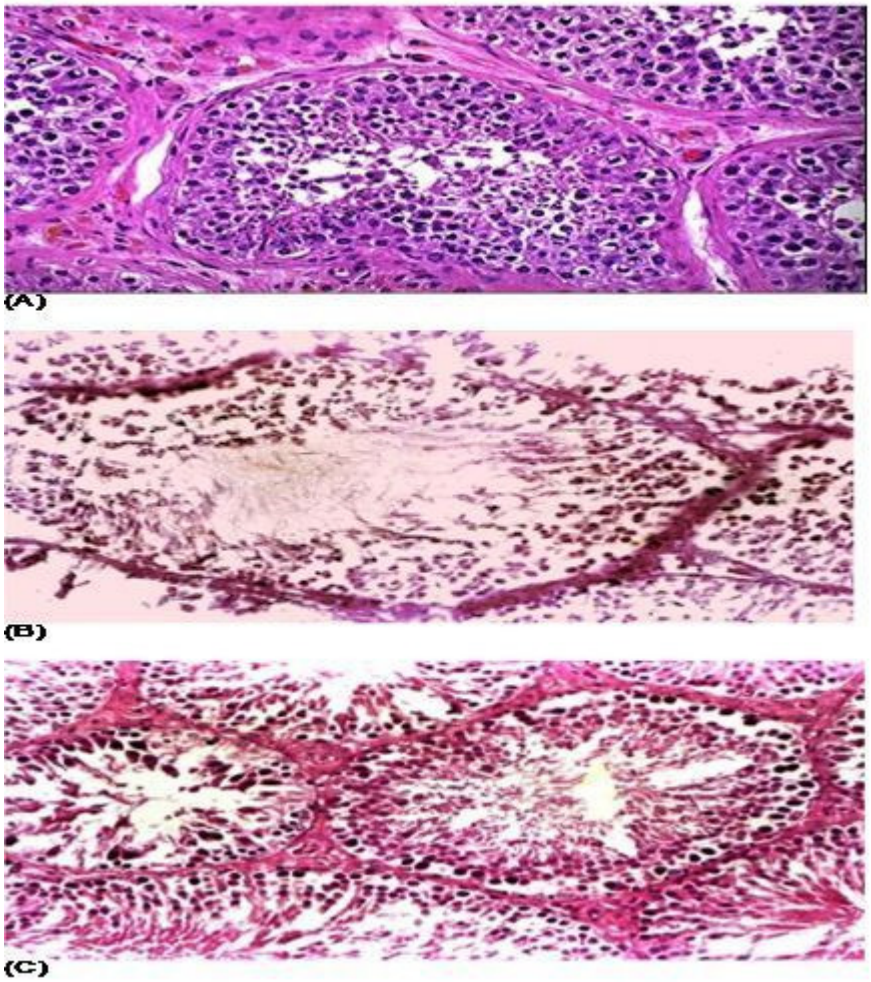

Figure 2. H&E stain. (A) Representative section of control, vehicle and DMH (200 mg/kg) group showing normal architecture of the seminiferous tubuls. (B) Section from the CCl_4 group (1 ml/kg; 10% in olive oil) showing deterioration of seminiferous tubules; germinal layers, basement membrane is absent and seminiferous tubule is infiltrated with the inflammatory cells. (C) Section from DMH (CCl_4+200 mg/kg) group showing the near normal structure of seminiferous cells; basement membrane and germinal layers are well developed.

compared to the control group. In accordance with this result, the examination by light microscopy has shown a diffuse, microvesicular fatty infiltration in CCl_4 group. Most of the histological abnormalities disappeared in the corresponding groups receiving the DMH at two high doses (150 and 200 mg/kg body weight), indicate the stabilization of plasma membranes as well as repair of hepatic tissue damages caused by CCl_4. In contrary to the low level of LDL, serum level of TCL and HDL were significantly increased with CCl_4 induced toxicity in this study. Treatment of DMH in the diet reversed the effects of CCl_4 treatment towards the normal level indicating the ameliorating effects of DMH which were more pronounced at two high doses of DMH (150 and 200 mg/kg body weight).

The free radicals and its triggered lipid peroxidation were involved in the main mechanisms by which carbon tetrachloride injured hepatocytes (Sreelatha et al., 2009). In this study CCl_4 treatment inhibits the activities of antioxidant enzymes; CAT, POD, SOD, GST, GSH-Px, GSR and QR and depletes the cellular GSH contents. Szymonik-Lesiuk et al. (2003) have shown that CCl_4 intoxication can lead to alteration in gene expression and depletion of CAT and SOD levels in liver. The CAT, POD and SOD activity was brought to increase by the treatment of DMH to CCl_4-treated rats. Treatment of various doses of DME possibly inhibited the conversion of CCl_4 into its reactive metabolites, decreased the oxidative stress and protected the antioxidant enzymes of liver as revealed by the enhanced level of CAT, POD and

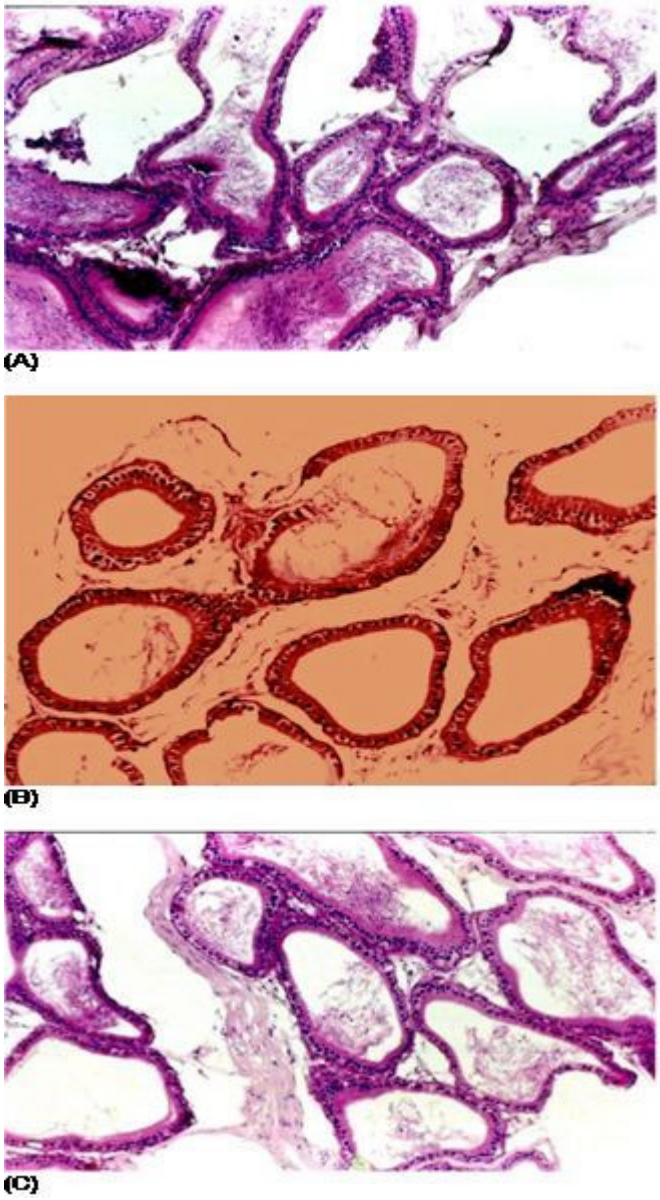

Figure 3. H&E stain. (A) Representative thin section from control, vehicle and DMH (200 mg/kg) group showing normal structure of the epididymis with sperms. (B) Section from CCl_4 (1 ml/kg; 10% in olive oil) group showing degeneration and scanty lumen of epididymis. (C) Section from DMH (CCl_4+200 mg/kg) showing the well organized epididymis with sperms in the lumen.

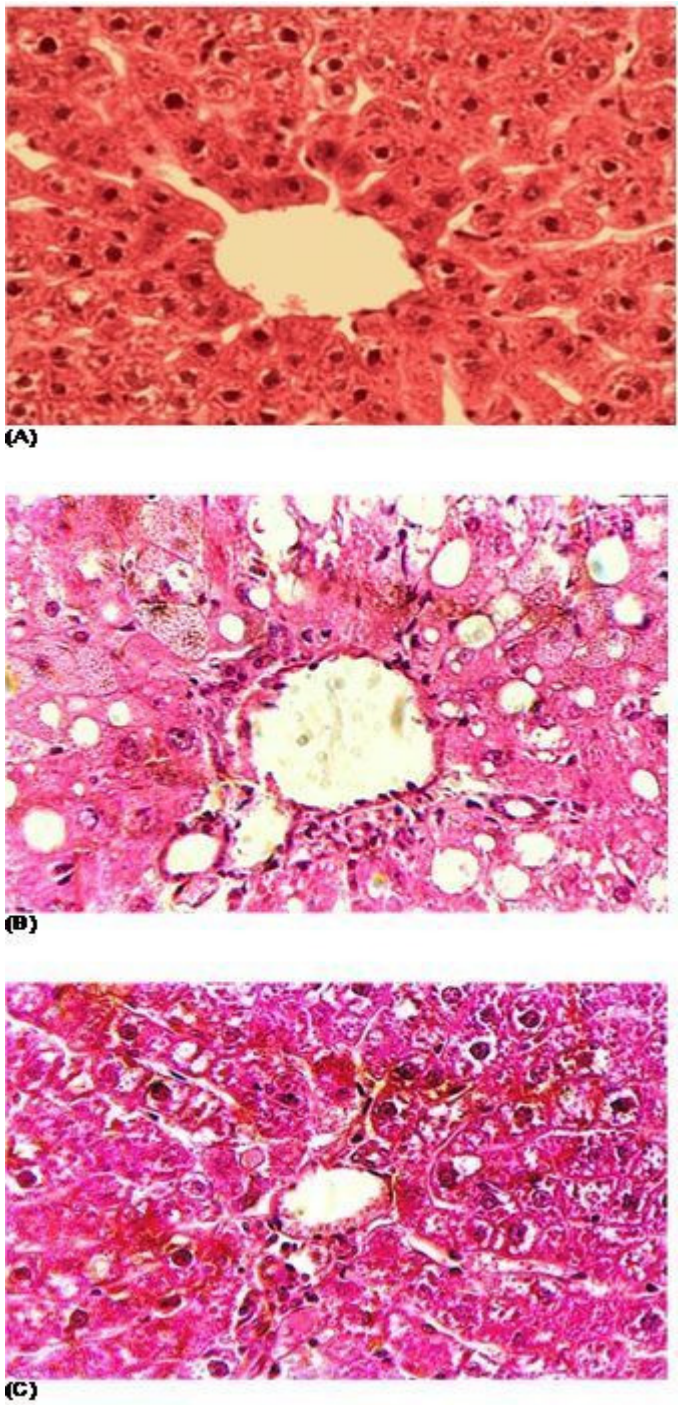

Figure 4. H&E stain. (A) Representative section from control, vehicle and DMH (200 mg/kg) depicting the normal structure of lobule and hepatocytes. (B) Section from the CCl_4 (1 ml/kg; 10% in olive oil) showing the centrilobular degeneration and fatty infiltration in hepatocytes. (C) Section from the DMH (CCl_4+200 mg/kg) group showing the near normal architecture of the lobule and hepatocytes.

SOD in this experiment.

GSH is an important protein thiol which coordinates body defense system against oxidative stress (peroxide scavenger), could eliminate superoxide anion and hydrogen peroxide. The maintenance of sufficient glutathione levels is important for the prevention of CCl_4-induced damages. Reduced glutathione (GSH) effectively scavenge free radicals and other reactive free oxygen species. In such reactions, GSH is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent glutathione reductase. We obtained significant depletion of GSH and GSH-Px while increase in the TBARS and nitrite contents as compared to the control

group in this experiment (Ohkawa et al., 1979). Nitrites can maintain tissue protective reactions as well as pro-oxidant effects. Nitrite reacts with superoxide radical ($\cdot O_2$) result in peroxynitrite which is a strong oxidant that reacts with thiols and initiates lipid peroxidation (Rubbo et al., 1996; Khan et al., 2009; Khan et al., 2010).

We hypothesize that DMH would be able to protect CCl_4-induced deleterious effects in rat liver as a result of its intrinsic ameliorating properties. The results of the present study demonstrate that DMH caused marked amelioration of CCl_4-induced deleterious effects by normalizing the enzymes of phase II that is GSR, GSH-Px, GST and other metabolic enzymes QR and γ-GT. The antioxidant activity or inhibition of the generation of free radicals is important in the protection against CCl_4-induced liver lesion (Sreelatha et al., 2009). Chemical compounds such as rutin and hyperoside determined in DMH have been reported to exert antioxidant activity by scavenging free radicals that cause lipid peroxidation (Yuan et al., 2008).

In the present study, CCl_4-induced testicular txicity was identical to that previously reported in rat (Khan and Ahmed, 2009). The study herein poresented was instigated by earlier study that CCl_4 administration caused testicular atrophy, degeneration of germinal layer, decrease in testosterone, gonadotropins (FSH, LH) and increase in prolactin and estradiol in male rat (Khan and Ahmed, 2009). This action of CCl_4 on the testis may be ascribed a direct toxic action of CCl_4 on the tissues and is likely to impair gonadal response to FSH and LH; diminished production of testosterone. In addition, liver diseases in human are associated with several hormone disorders, including decreased serum levels of T_3, cortisol, testosterone, FSH and insulin, and elevated prolactin concentrations in males (Barreca et al., 1983; Kolster et al., 1990).

CCl_4 might affect the suprachiasmatic hypothalamic nucleus (SCN) which regulates the pituitary hormone secretions. Increase in serum level of prolactin and estradiol might be related with dysregulation of pituitary function as an insult of CCl_4. The increase in testosterone, FSH and LH level in this experiment with administration of DMH could be attributed to its direct effect on the central nervous system and gonadal tissues or their effects on hypothalamus-pituitary-testis axis.

In the current study, CCl_4 administration for 16 weeks induced a decrease in weights of accessory sex organs. The decrease in these reproductive organs weights could be due to the decrease in testosterone level as result of oxidative damage (Khan and Ahmed, 2009) and the histological alterations induced by CCl_4 in testis and liver. The histopathological findings of testes in the CCl_4-treated group showed a decrease in size and germinative cell layer, thickness of seminiferous tubules suggesting spermiotoxicity through oxidative damage to biomolecules. Histopathology of epididymis also shows alterations in the structure with scanty secretions in the lumen and deprived of sperms. Interestingly, DMH normalize the histological alterations induced by CCl_4 treatment.

Conclusion

Results obtained in the present study suggested the protective potential of DMH against the CCl_4-induced liver and testicular toxicity. DMH treatment ameliorated the hepatic injuries with consequent increase in the antioxidant status of various enzymes and compounds. Level of testosterone was elevated with DMH in addition to the repairing of testis and accessory organs. These protective effects of DMH against the CCl_4 toxicity may be attributed due to the presence of various bioactive groups and specifically the rutin and hyperoside in DMH.

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