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Red wine ameliorates CCl_4 – induced acute liver injury in rats

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Abstract

Consumption of a naturally occurring polyphenol, in moderate amounts, has been suggested to be beneficial to health. The present study investigated the efficacy of red wine on prevention of carbon tetrachloride (CCl_4) induced acute hepatic injury in wistar rats. Hepatic damage due to CCl_4 intoxication was assessed by employing biochemical parameters, markers of hepatic oxidative damage were measured in terms of ROS, MDA, GSH and enzyme antioxidants (CAT, SOD, GPx) levels. Immune parameters (IgG1, IgG2, CD4, CD8), cytokines (IL-6, IL-10) and gene expressions of COX-2 and CYP2E1 were carried out. In addition, CCl_4-induced pathological changes in liver were evaluated by histopathological studies. Pre-treatment with red wine significantly prevented increase in serum hepatic enzymatic activities, ROS, MDA levels as well as depletion of glutathione. Pre-treatment of red wine normalized the antioxidant enzyme activities in CCl_4 intoxicated rats. Our data revealed that pre-treatment with red wine normalized the levels of interleukin 6 &10, IgG and CD4 cell count. Pre-treatment of red wine significantly prevented COX-2 inflammatory response and the up regulation of CYP2E1 expression as well. Histopathological findings also revealed the protective effect of red wine. Our findings provide evidence to demonstrate that red wine has a potent hepato-protective effect on CCl_4-induced liver injury in rats through its antioxidative, immuno-modulatory and anti-inflammatory activity.

Keywords: Carbon tetrachloride, Red wine, Oxidative stress, Cycloxygenase-2, CYP2E1
Abbreviations:ROS: Reactive oxygen species, MDA: Malondialdehyde, GSH:Glutathione, CAT: Catalase, SOD: Superoxide dismutase, GPx Gluathione peroxidase, DTNB:5, 5 □-dithiobis-2-nitrobenzioc acid, DCFDA: Dichlorofluorescin diacetate, COX: Cycloxygenase, CCl_4:Carbon tetra chloride , IL: Interleukins, IgG: Immunoglobulin G.

Introduction

Inflammation is an important protective response to cellular injury, which destroy and remove the injurious agent and injured tissue, thereby promoting tissue repair. When this crucial and normally beneficial response occurs in an uncontrolled manner, the result is excessive cellular damage which causes chronic inflammation and destruction of normal tissue by enhancing oxidative stress. Reactive oxygen species(ROS) such as the superoxide anion liberated by phagocytes recruited to sites of inflammation are proposed to be a major cause of the cell, tissue damage and is associated with many chronic inflammatory diseases [1-2]. Hepatocytes are susceptible to the injurious effects of oxidants when exposed to various toxic substances like carbon tetrachloride (CCl_4), ethanol and acetaminophen [3]. CCl_4 is one of the chlorinated hydrocarbons that have a wide spread use in various industries as a solvent and also used in medicine as a vermifuge in treatment of hookworm disease [4, 5]. Prolonged administration of CCl_4 causes increase in concentration of serum hepatic enzymes and also leads to fibrosis, cirrhosis and hepatic carcinoma [6]. CCl_4 intoxification causes activation of immune cells which subsequently infiltrate damaged liver; these immune cells then secrete inflammatory mediators such as COX-2, interleukins and cytokines/ chemokines in response to oxidative stress. Dietary antioxidants have been proposed as therapeutic agents to counteract oxidative stress. Natural antioxidants may act as protectors of several pathologies not only as conventional hydrogen-donating compounds (antiradical activity) but, more importantly, may exert modulatory effects in cells through actions in antioxidant, drug metabolizing and repairing enzymes as well as working as signaling molecules in important cascades for cell survival [7-9]. Polyphenols are the largest group among natural antioxidants and red wine has attracted particular interest due to a high content of biologically active compounds and has found to possess anticancer, anticardio-vascular disease and anti-inflammatory activities [10, 11]. Thus, the present study was designed to investigate the antioxidant, immunomodulatory and anti-inflammatory potential of red wine against CCl_4 induced hepato-toxicity in Wistar rats.

Table 1. Effect of red wine pre-treatment on body weight, relative liver and spleen weight in CCl_4 intoxicated rats.

Group	Body weight Mean ± SD, *n*=10	Relative organ weight (g/100g of b.w.) Mean ± SD, *n*=10	
		Liver	**Spleen**
Control	208 ± 20	2.5 ± 0.8	0.12 ± 0.01
CCl_4	156 ± 26**	4.3 ± 0.5**	0.36 ± 0.08**
CCl_4 + RW	198 ± 14*	2.9 ± 0.4*	0.19 ± 0.04*
RW	206 ± 16$^{\#}$	2.4 ± 0.5$^{\#}$	0.13 ± 0.02$^{\#}$

Values are expressed as mean ± SD, **-Significantly different from the control ($p < 0.01$), *-Significantly different from the CCl4-treated ($p < 0.05$), # -Not significantly different from the control ($p > 0.05$)

Results

Body and organ weight of rats

Table 1 shows the body, liver and spleen weight of rats in each group. A decrease in body weight and increase in liver and spleen weight was observed in CCl_4 treated rats than those of control group. Pre-treatment with red wine (500µL/kg b.w/day) for 8 weeks significantly protected the decrease in body weight and increase in liver weight when compared with CCl_4 treated rats.

Serum biochemistry: Indices of hepatotoxicity and protection

The serum hepatic enzyme levels, such as ALP, AST, ALT, LDH and Total bilirubin were significantly elevated in CCl_4 treated rats compared with control group. Pre-treatment of red wine for 8 weeks significantly prevented the elevation of serum hepatic enzymes as compared with those of CCl_4 treated group (Table 2).

Effect of red wine on hepatic oxidative stress

The generation of ROS in rat liver was found to be significantly increased ($p< 0.001$) in CCl_4 treated group than in controls. However, pre-treatment with red wine significantly prevented ($p< 0.001$) the generation of ROS compared with control group (Fig1).

As shown in inset of figure 1, the hepatic MDA in CCl_4 treated rats increased significantly ($p<0.001$) compared with the controls, while pre-treatment with red wine significantly prevented ($p= 0.006$) the increase in hepatic MDA level. Significant ($p<0.001$) reduction of hepatic GSH was observed in CCl_4 treated rats when compared with control group. Whereas, pre-treatment with red wine significantly ($p<0.001$) protected hepatic GSH from reduction (inset b Fig1).

Effect of red wine on antioxidant enzyme activities

Table 3 shows the protective effect of red wine against CCl_4 - induced alteration of antioxidant enzyme in Wistar rats. CCl_4 administration alone significantly altered the antioxidant enzymes (CAT, GST, SOD and GPx) activities, whereas pre-treatment with red wine significantly protected the alteration of CAT ($p=0.02$), GST ($p=0.008$), SOD ($p=0.03$) and GPx ($p=0.001$).

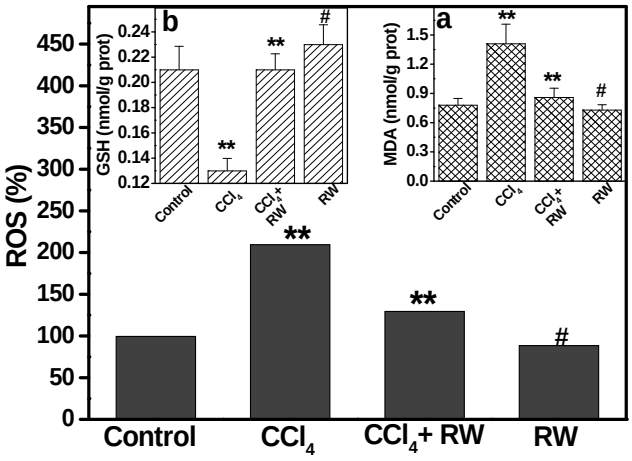

Fig 1. Inhibition of CCl_4 induced ROS formation by pre-treatment of red wine. Insets shows (a) Effect of red wine on hepatic lipid peroxidation (b) Effect of red wine on cellular glutathione. Values are expressed as mean ± S.D, **Significantly different from the control; p <0.01, # Significantly different from the CCl_4 treated; p <0.05

Immune and cytokine levels

Table 4 shows the immune and cytokines levels. CD4 T-lymphocyte count and CD4/CD8 ratio were significantly decreased ($p=0.04$) in CCl_4 treated rats, however no change in the CD8 count was observed. The serum levels of IgG1 and IgG2 were found to be significantly increased ($p<0.001$) in CCl_4 treated rats with respect to controls. An increase in the levels of IL-6 and IL-10 cytokines was observed in CCl_4 treated rats (289 ± 32pg/mL, 761 ± 98 pg/mL) than controls (212 ± 84 pg/mL and 476 ± 32 pg/mL). However, pre-treatment with red wine significantly prevented the elevations.

Expression of COX-2 and CYP2E1

A significant up-regulation of COX-2 and CYP2E1 mRNA expression was observed in CCl_4 treated rats with respect to controls. Pre-treatment with red wine attenuated the over expression of COX-2 and CYP2E1 in treated rats (Fig 2).

Table 2. Effect of red wine pre-treatment on CCl_4 induced alteration in serum enzyme activities.

Group	AST (IU/L)	ALT (IU/L)	ALP(IU/L)	TBiL	LDH
Control	107.13 ± 12	43.86 ± 12	72.51 ± 14	0.8	465 ± 43
CCl_4	176 ± 32[a]	68 ± 13[a]	176.21 ± 19[a]	1[c]	543 ± 89[a]
CCl_4 + RW	87 ± 12[b]	35.65 ± 15[b]	64.21 ± 12[b]	0.9[c]	476 ± 76[b]
RW	101.38 ± 11[c]	40.22 ± 8[c]	71.11 ± 10[c]	0.8[c]	460 ± 6[c]

Values are expressed as mean ± SD, a-Significantly different from the control ($p < 0.01$), b-Significantly different from the CCl4-treated ($p < 0.05$), c -Not significantly different from the control ($p > 0.05$)

Histopathological studies

Sections of liver stained by H&E were examined for necrotic cells, inflamed cells, neutrophils infilteration, cellular hypertrophy and fatty infilteration. In CCl_4 administered rats, the hepatic injury was marked and widespread with fatty changes, cellular hypertrophy and necrotic foci while pre-treatment with red wine significantly attenuated CCl_4-induced liver damage (photographs not shown).

Discussion

Food rich in plant bioactive compounds and polyphenols in particular may exert beneficial effects towards human health. Polyphenols are the largest group among natural antioxidants, about 8000 compounds that includes mainly flavonoids, phenolic acids, lignans, coumarins, tannins, xantans and chromons. Plant polyphenols are non-nutritive, hydrophilic components found in small amounts (micrograms) in all kind of plant-derived food sources such as fruits and vegetables, drinks (wine, coffee, juices) and cereals. Among natural antioxidants red wine has attracted particular interest due to a high content of biologically active compounds. It has been considered to play an important antioxidant role as dietary antioxidant for prevention of oxidative damage in living system [20].

In this study we evaluated immuno-modulatory and anti-inflammatory effect of red wine on CCl_4 intoxicated Wistar rats. CCl_4 was administered in rats as it is a widely used hepatotoxic agent and is known to enhance formation of free radicals through its metabolism and to subsequently cause lipid peroxidation of cellular and organelle membranes as a primary pathogenic step [21]. We observed a significant alteration in the body weight, liver and spleen weight of CCl_4 intoxicated rats than controls indicating that hepatic injury had been induced which is in concomitant with the previous published data [22].

Hepatic damage induced by CCl_4 administration is observed by evaluating LDH, AST and ALT levels in CCl_4 treated rats. Serum enzymes such as aspartate aminotransferase and alanine aminotransferase are employed in evaluation of hepatic disorders and an increase in these enzyme activities reflects acute liver damage and inflammatory hepatocellular disorders. The result of the present study illustrated that CCl_4 administration caused severe acute liver damage in rats, demonstrated by significant elevation of serum liver enzymes which are in consistent with the previous findings [23, 24]. However, pre-treatment with red wine markedly reduced the liver enzyme activities suggesting significant protection of the liver.

Lipid peroxidation is one of the principal causes of CCl_4 induced liver injury and is mediated by the free radicals derivatives of CCl_4. In consistent with the previous reports our data also revealed a substantial increase in ROS, MDA levels and depletion of GSH levels in CCl_4 intoxicated rats [25]. In addition, the antioxidant activity and or the inhibition of free radical generation are important for protecting the liver against CCl_4 induced damage [26]. We also reported decreased activities of CAT, SOD, GST and GPx as these enzymes are easily inactivated by lipid peroxides or ROS, which resulted in decreased activity of these enzymes. However, pre-treatment of red wine significantly reduced the levels of ROS, MDA and also prevented the depletion of GSH and antioxidant enzymes as well. This protective effect of red wine may be endorsed to its free radical scavenging activities with high oxygen radical scavenging capacities may be affected by oxygen pressure and the formation of active oxygen species during the oxidation of polyphenols [27]. The stronger antioxidant activity of red wine is also due to its higher phenolic compounds concentration which is 20 times higher in red wine when compared to white wine [28].

Cellular and humoral immune responses are essential for the prevention and defense against inflammation and toxicity [29]. We observed a prominent increase in IgG levels in CCl_4 intoxicated rats when compared to controls. Nevertheless, pre-treatment with red wine significantly diminished the levels of IgG, which may be either due to the direct suppression of IgG production or due to nullification of CCl_4 toxicity. We also observed a considerable decrease in decrease in CD4 cell count representing immunotoxicity by CCl_4. Pre-treatment with red wine illustrated a marked increase in CD4 count, which reveals an immunomodulatory effect of red wine. However, we could not find any change in the CD8 count either in CCl_4 or in red wine treated rats.

Cytokines like IL-6 and IL-10 play a potential role in the pathogenesis of liver diseases [30]. In the present study, levels of IL-6 and IL-10 were consistently found to be elevated in CCl_4 intoxicated rats than in controls. Pre-treatment of red wine strengthen the immune response towards CCl_4 toxicity by normalizing their levels.

Cyclooxygenase-2 (COX-2) is an inducible, rate-limiting enzyme responsible for production of large amount of prostaglandins (PG), especially when activated by stimuli, such as inflammation, cytokines and lipopolysaccharides [31]. Our data showed over expression of COX-2, synthesis of which is up regulated by inflammatory stimuli or hyper oxidation of lipid, induces synthesis of PG, which causes liver injury. Whereas, pre-treatment with red wine significantly reduced the inflammation, confirming the anti-inflammatory activity of red wine.

CCl_4 becomes toxic upon activation mainly through CYP2E1, and an induction or an over-expression of this cytochrome correlates with higher CCl4 toxicity [32, 33]. After CCl_4 bioactivation, the resulting $CCl_3^{\bullet}$ radical binds covalently to CYP 2E1, either to the active site of the enzyme or to the heme

Table 3. Effect of red wine pre-treatment on CCl_4 induced alteration in antioxidant enzyme activities.

Group	CAT (µmol/min/mg prot)	GST (µmol/min/mg prot)	SOD (Units/mg prot)	GPx (nmol/min/mg prot)
Control	232.43 ± 13.2	1.47 ± 0.23	21.54 ± 1.53	278.63 ± 31.76
CCl_4	186.25± 9.2[a]	1.11± 0.53[a]	29.65 ± 2.11[a]	213.43 ± 53.12[a]
CCl_4 + RW	226.33± 18.8[b]	1.38± 0.51[b]	23.24 ± 3.83[b]	245.76 ± 11.96[b]
RW	238.66 ± 8.9[c]	1.58 ± 0.22[c]	19.88 ± 5.66[c]	270.11 ± 22.36[c]

Values are expressed as mean ± SD, a-Significantly different from the control ($p < 0.01$), b-Significantly different from the CCl_4-treated ($p < 0.05$), c -Not significantly different from the control ($p > 0.05$)

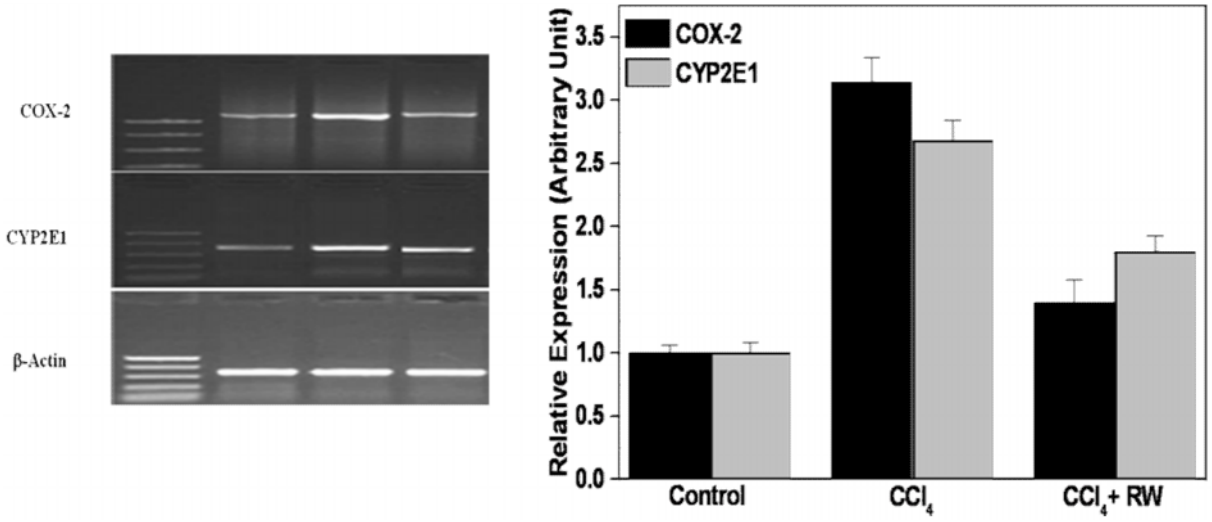

Fig 2. Effect of red wine on expression of COX 2 and CYP2E1 in CCl_4- induced toxicity. β actin was used as an internal control.

group, thereby causing suicide inactivation [32]. CCl_4 is mainly metabolized to highly reactive trichloromethyl radicals by CYP2E1 [32]. These reactive free radicals induce liver damage by triggering a chain of cellular events [34]. Our data showed a significant increase in the expression of CYP2E1 in CCl_4 treated rats. Nevertheless, the pre-treatment with red wine significantly prevented the over expression of CYP2E1. This result suggests that the suppression of CYP2E1 is an important aspect of the hepato-protective effect of red wine.

Histopathological studies showed typical fibrotic appearances with marked degeneration of hepatocytes in the liver tissues of CCl_4 treated rats compared with controls. However, in red wine pre-treated rats, cell injury coagulative necrosis inflammatory infilteration granulocytes and mononuclear inflammatory cells were occasionally observed and CCl_4 induced lesions were markedly prevented as well. These appearances are generally in agreement with previous studies performed using antioxidants and hepatotoxic substances [35, 36]. The results obtained from our study suggest that red wine can diminish liver fibrosis induced by CCl_4.

Materials and methods

Chemicals

Red Wine was obtained from local supermarket and wine shops. Thiobarbituric acid (TBA), butylated hydroxy toluene (BHT) and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were of the best purity available and obtained from local agents. CCl_4 was obtained from Merck, Germany. All the other chemicals were of analytical grade. Solutions were prepared in nano-pure water.

Animals and experimental design

A total of 40 adult male Wistar rats weighing 180-200g were used. The rats were housed in macrolon cages under standard laboratory conditions (12h light/ dark cycle, 23 ± 2°C). Animals received human care according to the criteria outlined in the "Guidelines of animal ethics committee with the Indian National law on animal care and use".

Table 4. Effect of red wine pre-treatment on immune cells, IgG and interleukins in CCl_4 induced toxicity.

Group	CD4	CD8	CD4/CD8	IgG_1 (%)	IgG_2 (%)	IL-6 (pg/mL)	IL-10 (pg/mL)
Control	32	8	4.00	100	100	212 ± 84	476 ± 32
CCl_4	26	9	2.88	145	168	289 ± 32	761 ± 98
CCl_4+RW	31	8	4.37	112	118	196 ± 13	454 ± 32
RW	33	9	-	100	98	180 ± 28	389 ± 41

The rats were randomly allotted into four experimental groups, each containing 10 rats. The first group served as master control without any treatment. The second group was treated with CCl_4 (10μL/kg) b.w, intraperitoneally (i.p) dissolved in corn oil (2% v/v) twice a week for consecutive 8 weeks. Animals in the third group were pre-treated with red wine (500μL/kg b.w for 8 weeks i.p) with total phenolic content 2912mg/L of gallic acid equivalent and CCl_4 (20μL/kg b.w, twice a week for 8 weeks). Fourth group served as red wine master control (500μL/kg b.w for 8 weeks).

On the 60^{th} day, blood was drawn out for the analysis of biochemical parameters and gene expressions and the animals were sacrificed by cervical dislocation. The liver was excised rapidly and assayed for GSH level, GSH-dependent enzymes activity, MDA formation and histopathology.

Serum Biochemistry analysis

ALT, AST, ALP, Tbil and LDH were measured using spectophotometric diagnostic kits (Sigma chemical Co, St. Louis, MO) as described previously [12]. Briefly, the samples were centrifuged at 1000 X g for 10 min within 1 hour after collection. The sera were stored at –80°C freezer before analysis. Enzyme activity in blood serum was evaluated by an autoanalyzer (ShimadzuCL-7200, Shimadzu, Japan).

Biomarkers of Oxidative stress

The oxidative damage was evaluated by estimating ROS, MDA and GSH levels. ROS production was quantified by the Dichlorofluorescin diacetate (DCFH-DA) method [13] based on the ROS– dependent oxidation of DCFH-DA to DCF according to the method described elsewhere [14]. Lipid peroxidation was estimated using thiobarbituric acid reactive substance (TBARS) assay as reported previously [15]. Results were expressed as nmol/g protein. Cellular protein thiols levels were measured using dinitro thio benzoic acid (DTNB) and expressed as nmol/g protein using standard GSH.

Analysis of CD4 and CD8 cells

Quantification of CD4, CD8 cells were carried out by flowcytometer FACS Calibur (Becton & Dickinson, San Diego CA, USA). Briefly, 20μL of the antibody and 50μL of the anticoagulated blood were incubated in 5mL polystyrene round-bottomed tube (Cat. No. 352003, Becton & Dickinson, San Diego, USA), for 15-20min. Then the cells were lysed using 1X FACS lysing solution (BD FACS lysing solution, Cat. No. 349202 Becton & Dickinson, San Diego CA, USA). The tubes were further incubated in dark for 10-12min and washed twice with 2mL sheath solution (BD FACSFlow Cat. No. 342003, Becton & Dickinson, San Diego CA, USA) subsequently to remove the debris. Finally the precipitate was resuspended in 450μL of the sheath solution for flowcytometric analysis. Acquisition and analysis of the processed samples were performed using CELLQuest software (Becton & Dickinson, San Diego CA, USA) [16].

Analysis of IgG, IL-6 and IL-10 in blood

A commercial enzyme linked immunosorbent assay kit (ELISA, Helori-test® Eurospital, Trieste Italy) was used to analyze IgG antibodies in serum. Plasma levels of IL-6 and IL-10 were determined by ELISA (R&D Systems, MN) [17].

Analysis of antioxidant enzymes

Catalase (CAT), Glutathine-S-transferase (GST), Superoxide dismutase (SOD) and Gluathione peroxidase (GPx) assays were performed according to the instructions described previously [18]. Briefly, catalase activity was defined as the amount of enzyme required to decompose 1μM H_2O_2 in 1 min. The reaction was initiated by addition of 1.0ml of freshly prepared 20mM H_2O_2. The rate of H_2O_2 decomposition was measured spectrophotometrically at 240nm for 1 min. To measure SOD activity, xanthine and xanthine oxidase were used to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride to form a red formazan dye. Absorbance was then measured at 550nm. Hepatic GST activity was measured in 0.1M phosphate buffer (pH 6.5), 1mM GSH and 1mM 1-chloro-2, 4-dinitrobenzene in a 50μL sample. Increases in the absorbance were monitored for 10 min at 25°C at a wavelength of 340nm. GPx was assayed by the reaction between glutathione remaining after the action of GPx and 5, 5 □-dithiobis-(-2-nitrobenzioc acid) to form a complex that absorbs maximally at 412nm.

Gene expression of COX-2 and CYP2E1

COX-2 and CYP2E1 gene expressions were evaluated semi quantitatively by reverse transcriptase PCR (RT-PCR) according to the previous report [19]. The COX-2 and CYP2E1 expression levels were measured using Quantity one software (Bio-Rad, Hercules, CA, USA) by comparing with β-actin.

Histopathological assessment for liver damage

Fresh liver tissues that had been trimmed to a thickness of 3mm were placed in plastic cassettes and immersed in neutral buffered formalin for 24 hrs. Fixed tissues were processed routinely, and then embedded in paraffin, sectioned, deparaffinized and rehydrated using standard techniques. The extent of CCl_4 induced hepatotoxicity was evaluated by

assessing the morphological changes in liver sections stained with hematoxylin and eosin (H &E).

Statistical Analysis

The difference between control and study group was evaluated using Chi (χ^2) square test and Fisher exact test. The oxidative stress, inflammatory and immune parameters were analyzed using Two-tailed Student's t-test and Fisher's exact test. Analysis of all the statistical data were performed using SPSS version 13.0 and Origin 8.0 Pro. *p*-value <0.05 was considered as statistically significant.

Conclusion

The present study suggests that red wine has a potent hepatoprotective activity in CCl_4- induced liver injury in rats. This preventive effect of red wine is due to its free radical scavenging, antioxidative, immuno-modulatory and anti-inflammatory properties.

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