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Hepatoprotective and antioxidant effect of corosolic acid on carbon tetrachloride induced hepatotoxicity

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The present study was designed to investigate the hepatoprotective and antioxidant properties of corosolic acid (CRA) on carbon tetrachloride (CCl_4)-induced liver damage in rats. Liver damage was induced by giving a single oral dose of CCl_4 (1:1 in liquid paraffin) at 1.25 ml/kg body weight (BW). Rats were pretreated with CRA dose of 10, 20 and 40 mg/kg BW (once daily for 7 days before CCl_4 intoxication). Pretreatment with CRA showed significant hepatoprotection by reducing the aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) enzymatic activities which had been raised by CCl_4 administration. The levels of lipid peroxidation markers such as thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) were significantly increased by CCl_4 administration and pretreatment with CRA; the levels of lipid peroxidative markers were reduced. The activities of enzymic antioxidants (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) and the levels of non enzymic antioxidants (Vitamins C, Vitamins E and reduced glutathione (GSH)) were decreased by CCl_4 administration and those pretreated with CRA above enzymic and non enzymic antioxidants were increased. The present study concluded that CRA possesses hepatoprotective and antioxidant properties against CCl_4-induced hepatotoxicity in rats.

Key words: Hepatotoxicity, carbon tetrachloride (CCl_4), corosolic acid, lipids peroxidations, antioxidant.

INTRODUCTION

The liver is a vital organ present in vertebrates and some other animals. It has a wide range of functions such as drug metabolism, amino acid metabolism, lipid metabolism and glycolysis. Hepatotoxic chemicals cause the liver damages which are induced by lipid peroxidation and other oxidative damages (Muhtaseb et al., 2008; Appiah et al., 2009). Carbon tetrachloride (CCl_4), a well-known model compound for producing chemical hepatic injury and it is biotransformed by hepatic microsomal cytochrome P450 (CYP) 2E1 to trichloromethyl-free radicals (CCl_3• and/or CCl_3OO•) (Brattin et al., 1985; Rechnagel and Glende, 1973; Rikans et al., 1994). Generally, these metabolites react with antioxidant enzymes such as glutathione (GSH) and catalase and superoxide dismutase (SOD) (Rikans et al., 1994). However, overproduction of trichloromethyl-free radicals is considered the initial step in a chain of events that eventually lead to membrane lipid peroxidation and finally to cell apoptosis and necrosis (Basu, 2003; Brautbar and Williams, 2002; Weber et al., 2003; Williams and Burk, 1990).

Modern medicines have little to offer for alleviation of hepatic diseases and it is chiefly the plant based preparations which are employed for the treatment of liver disorders (Somasundaram et al., 2010). There is a great demand for the development of an efficient hepatoprotective drug from the natural resource (Tandon et al., 2008). Corosolic acid (CRA), a triterpenoids, which is isolated from *Actinidia valvata* Dunn and also has been discovered in many Chinese medicinal herbs, such as the *Lagerstroemia speciosa* L (Fukushima et al., 2006) and banaba leaves (Yamaguchi et al., 2006). It has been

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reported that CRA produces an excellent anti-diabetic activity in some animal experiments and clinical trials, including improvement of glucose metabolism by reducing insulin resistance in a mice model and lowering effect on post-challenge plasma glucose levels in human (Fukushima et al., 2006; Miura et al., 2006). It was also reported that CRA displayed some cytotoxic activities against several human cancer cell lines (Ahn et al., 1998; Yoshida et al., 2005). In the present study, we investtigated the hepatoprotective and antioxidant properties of CRA used against CCl_4-induced liver damage in rats.

MATERIALS AND METHODS

Chemicals

CCl_4 was purchased from Sigma-Aldrich Co., St. Louis, Missouri, USA. CRA was purchased from Mansite Pharmaceutical Co., Ltd (Chendu, China). All other chemicals used were of analytical grade obtained from E. Merck or HIMEDIA, Mumbai, India.

Experimental animals

Male albino Wistar rats (180 to 200 g) were housed in clean cages at a 20 to 24°C temperature, 12-h light/12-h dark cycle and 52% relative humidity in the animal house at the College of Medicine, King Saud University. Ethics approval was obtained from the ethics committee of the College of Medicine Research Center at King Saud University, Riyadh, Saudi Arabia (11/3215/IRB). The animals were given free access to water and received a standard pellet diet. Animals care was perform in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH, 1985).

CCl_4-induced hepatotoxicity

Hepatotoxicity was induced a single oral dose of CCl_4 (1:1 in liquid paraffin) at 1.25 ml/kg BW at an interval of 6 h after the administration of last dose of CRA on the 7th day.

Experimental design

Dose determination study

The animals were divided into six groups of six animals in each group. CRA was suspended in 0.1% dimethylsulfoxide (DMSO), and fed to rats via an oral route at 10, 20 and 40 mg/kg body weight (BW) for 7 days. Takagi et al. (2010) reported that CRA treated mice at 10 mg/kg BW inhibit hypercholesterolemia and hepatic steatosis caused by dietary cholesterol in KK-Ay mice. Hence these three different doses were fixed based on previous report. Then a single oral dose of CCl_4 (1:1 in liquid paraffin) at 1.25 ml/kg BW was given at an interval of 6 h after the administration of last dose of CRA. Group I served as control rats that received 0.1% DMSO only, Group II served as control rats treated with 40 mg/kg BW CRA. Group III was administered CCl_4 (negative control). Groups IV, V and VI were administered CRA at 10, 20 and 40 mg/kg BW and also administered CCl_4 at an interval of 6 h after the administration of last dose of CRA on the 7th day. Animals were sacrificed after 24 h of CCl_4 administration. Blood was collected in a dry test tube and allowed to coagulate at ambient temperature for 30 min. Serum was separated by centrifugation at 2000 rpm for 10 min and used for estimated hepatic marker enzymes. Among the three different doses 20 mg/kg BW showed the maximum activity as compared to other two doses. So, the 20 mg/kg BW was fixed as an optimum dose and used for further study.

Experimental protocol for further study

The animals were divided into six groups of six animals in each group. CRA was suspended in 0.1% DMSO, and fed to rats via an oral route at 20 mg/kg BW for 7 days. Then a single oral dose of CCl_4 (1:1 in liquid paraffin) at 1.25 ml/kg BW (Saba et al., 2010) was given at an interval of 6 h after the administration of last dose of CRA. Group I served as control rats received 0.1% DMSO only, Group II served as control rats treated with 20 mg/kg BW CRA. Group III was administered CCl_4 (negative control). Groups IV was administered CRA at 20 mg/kg BW and also administered CCl_4 at an interval of 6 h after the administration of last dose of CRA on the 7th day. Animals were sacrificed after 24 h of CCl_4 administration. Blood sample was collected in tubes containing a mixture of ethylene diamine tetra acetic acid (EDTA) for the estimation of plasma lipid peroxidation and antioxidants. Tissue was sliced into pieces and homogenized in appropriate buffer in cold condition (pH 7.0) to give 20% homogenate. The homogenate were centrifuged at 1000 rpm for 10 min at 0°C in cold centrifuge. The supernatant was separated and used for various biochemical estimations.

Biochemical assays

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined in accordance with the method provided by Reitman and Frankel (1957) and King (1965a). The estimation of plasma and tissue thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) were done by the methods of Niehaus and Samuelson (1968) and Jiang et al. (1992), respectively. The activities of SOD, CAT and GPx in erythrocyte and tissue were measured by the methods of Kakkar et al. (1978), Sinha (1972) and Rotruck et al. (1973), respectively. The levels of vitamins C and E and GSH in plasma and tissue were estimated by the methods of Roe and Kuether (1943), Baker et al. (1980) and Ellman (1959), respectively.

Statistical analysis

Statistical evaluation was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using Statistical Package of Social Science (SPSS Inc., Chicago, IL, USA) 10.0 for Windows. Significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

The present study demonstrates the hepatoprotective, curative and antioxidant effects of CRA against CCl_4-induced liver injury in rats. Liver injury induced by CCl_4 is a common model for screening the hepatoprotective activity of drugs, because this chemical is a potent hepatotoxin and a single exposure can rapidly lead to severe hepatic necrosis and steatosis (Brautbar and Williams, 2002; Brent and Rumack, 1993; Manibusan et al., 2007). Table 1 shows the activities of hepatic function marker enzymes AST, ALT and ALP in the serum of control and CCl_4-induced hepatotoxicity in rats. Pretreat-

Table 1. Effect of CRA on the activities of hepatic marker enzymes in the serum of CCl_4-hepatotoxic rats.

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control	69.21 ± 4.11^{a}	36.13 ± 3.11^{a}	85.37 ± 6.69^{a}
Control + CRA (40 mg/kg BW)	70.36 ± 4.14^{a}	38.84 ± 2.71^{a}	86.39 ± 6.61^{a}
CCl_4-hepatotoxicity (1.25 ml/kg BW)	136.24 ± 10.51^{b}	108.16 ± 5.23^{b}	180.09 ± 12.18^{b}
CCl_4 + CRA (10 mg/kg BW)	106.68 ± 8.19^{c}	78.30 ± 5.39^{c}	136.23 ± 10.57^{c}
CCl_4 + CRA (20 mg/kg BW)	$75.54 \pm 5.51^{a,d}$	$42.16 \pm 3.10^{a,d}$	$90.15 \pm 6.20^{a,d}$
CCl_4 + CRA (40 mg/kg BW)	92.39 ± 7.99^{e}	63.26 ± 4.66^{e}	120.24 ± 10.77^{e}

Values are expressed as means ± standard deviation (SD) for six rats in each group. Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT).

Table 2. Effect of CRA on levels of TBARS and LOOH in plasma and liver of CCl_4-hepatotoxic rats.

Group	TBARS		LOOH	
	Plasma (mmol/dl)	Liver (mmol/100 g wet tissue)	Plasma (mmol/dl)	Liver (mmol/100 g wet tissue)
Control	0.20 ± 0.02^{a}	0.86 ± 0.05^{a}	6.60 ± 0.58^{a}	80.66 ± 5.15^{a}
Control + CRA (20 mg/kg BW)	0.22 ± 0.02^{a}	0.83 ± 0.05^{a}	7.10 ± 0.66^{a}	81.15 ± 3.18^{a}
CCl_4-hepatotoxicity (1.25 ml/kg BW)	0.56 ± 0.05^{b}	2.35 ± 0.15^{b}	28.47 ± 2.11^{b}	187.21 ± 10.20^{b}
CCl_4 + CRA (20 mg/kg BW)	0.31 ± 0.03^{c}	$0.94 \pm 0 .08^{c}$	10.19 ± 1.08^{c}	94.52 ± 6.88^{c}

Values are expressed as means ± standard deviation (SD) for six rats in each group. Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT).

ment with CRA showed significant hepatoprotection by reducing the AST, ALT and ALP enzymatic activities which had been raised by CCl_4 administration. Increases in serum AST, ALT and ALP levels by CCl_4 have been attributed to hepatic structural damage, because these enzymes are normally localized to the cytoplasm and are released into the circulation after cellular damage has occurred (Recknagel et al., 1989). The present study showed that pretreatment with CRA dramatically suppressed CCl_4-induced hepatic injury. We used at three different doses such as 10, 20 and 40 mg/kg BW of CRA and estimated hepatic marker enzymes. Among the three different doses, 20 mg/kg BW showed maximum activity and plays an important role in protecting against CCl_4-induced acute liver injury in rats. The lower dose of CRA (10 mg/kg BW) was not effective, because its concentration might not have been enough to counteract the CCl_4-induced toxicity. The higher concentration of CRA (40 mg/kg BW) might have resulted in the production of by-products that are interfering with the hepatoprotective activity, and consequently, decreasing its effect. Hence, 20 mg/kg BW of CRA is optimum for hepatoprotective activity. Hence, further study used optimum dose of 20 mg/kg BW only. Restoration of increased hepatic serum enzyme levels to normal levels reflects protection against the hepatic damage caused by hepatotoxins (Vogel, 2002).

The levels of TBARS and LOOH, respectively in the plasma and liver of control and CCl_4-induced heaptotoxicity in rats are shown in Table 2. The levels of lipid peroxidation markers such as TBARS and LOOH were significantly increased by CCl_4 administration and pretreatment with CRA, reduced the levels of lipid peroxidative markers. Lipid peroxidations as well as altered levels of some endogenous scavengers are taken as indirect *in vivo* reliable indices for oxidative stress (Comporti, 1987). The levels of lipid peroxidation in the CCl_4 treated rats were assessed by measuring the TBARS and LOOH in the liver tissue (Ganie et al., 2011). The increased TBARS and LOOH levels in the liver of CCl_4 treated animals indicate enhanced lipid peroxidation leading to tissue injury. CRA significantly lowered the levels of TBARS and LOOH could be related to its antioxidant capacity to scavenge reactive oxygen species (ROS). This result demonstrates the antiperoxidative and antioxidant effects of CRA. Drugs with antioxidant properties may supply endogenous defense systems and reduce both initiation and propagation of ROS (Bergendi et al., 1865).

We further studied the *in vivo* antioxidant activity of CRA by estimation of erythrocytes and liver. The activities of enzymic antioxidants (SOD, CAT and GPx) and non enzymic antioxidants (Vitamins C, Vitamins E and GSH) in the erythrocyte, plasma and liver of control and CCl_4-induced hepatotoxicity in rats are described in Table 3, 4, 5 and 6. The activities of enzymic antioxidants (SOD, CAT and GPx) and the levels of non enzymic antioxidants (Vitamins C, Vitamins E and GSH) were

Table 3. Effect of CRA on the activities of SOD, CAT and GPx in erythrocytes of CCl_4-hepatotoxic rats.

Group	Erythrocyte (U/mg Hemoglobin)		
	SOD	CAT	GPx
Control	6.82 ± 0.48^{a}	180.11 ± 13.15^{a}	14.58 ± 1.22^{a}
Control + CRA (20 mg/kg BW)	7.08 ± 0.50^{a}	179.23 ± 13.12^{a}	14.97 ± 1.32^{a}
CCl_4-hepatotoxicity (1.25 ml/kg BW)	3.32 ± 0.24^{b}	105.65 ± 8.45^{b}	6.29 ± 0.48^{b}
CCl_4 + CRA (20 mg/kg BW)	$5.92 \pm 0.34^{a,c}$	$176.63 \pm 10.31^{a,c}$	$13.\ 21 \pm 1.24^{a,c}$

U- Enzyme concentration required to inhibit the chromogen produced by 50% in 1 min under standard condition. U-μmole of H_2O_2 consumed/min. U-μg of GSH utilized/min. Values are expressed as means ± standard deviation (SD) for eight rats in each group. Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT).

Table 4. Effect of CRA on the activities of SOD, CAT and GPx in liver of CCl_4-hepatotoxic rats.

Group	Liver (U/mg protein)		
	SOD	CAT	GPx
Control	9.16 ± 0.72^{a}	85.32 ± 5.10^{a}	8.55 ± 0.56^{a}
Control + CRA (20 mg/kg BW)	8.96 ± 0.65^{a}	84.75 ± 4.86^{a}	8.23 ± 0.60^{a}
CCl_4-hepatotoxicity (1.25 ml/kg BW)	4.30 ± 0.35^{b}	39.09 ± 3.21^{b}	3.87 ± 0.34^{b}
CCl_4 + CRA (20 mg/kg BW)	8.31 ± 0.62^{a}	$79.21 \pm 4.99^{a,c}$	$7.22 \pm 0.51^{a,c}$

U- Enzyme concentration required to inhibit the chromogen produced by 50% in 1 min under standard condition. U-μmole of H_2O_2 consumed/min. U-μg of GSH utilized/min. Values are expressed as means ± standard deviation (SD) for eight rats in each group. Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT).

Table 5. Effect of CRA on the levels of GSH, vitamin C and vitamin E in plasma of CCl_4-hepatotoxic rats.

Group	Plasma (mg/dl)		
	GSH	vitamin C	vitamin E
Control	32.96 ± 1.88^{a}	3.12 ± 0.24^{a}	1.82 ± 0.10^{a}
Control + CRA (20 mg/kg BW)	32.04 ± 2.30^{a}	2.98 ± 0.26^{a}	$1.79 \pm .15^{a}$
CCl_4-hepatotoxicity (1.25 ml/kg BW)	16.24 ± 1.42^{b}	0.88 ± 0.05^{b}	0.48 ± 0.04^{b}
CCl_4 + CRA (20 mg/kg BW)	$27.30 \pm 2.45^{a,c}$	2.48 ± 0.21^{c}	1.35 ± 0.3^{c}

Values are expressed as means ± standard deviation (SD) for eight rats in each group. Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT).

Table 6. Effect of CRA on the activities of GSH, vitamin C and vitamin E in liver of CCl_4-hepatotoxic rats.

Group	Liver (mg/100 g wet tissue)		
	GSH	Vitamin C	vitamin E
Control	112.21 ± 10.45^{a}	0.86 ± 0.05^{a}	6.45 ± 0.51^{a}
Control + CRA (20 mg/kg BW)	111.19 ± 10.06^{a}	0.84 ± 0.05^{a}	6.32 ± 0.56^{a}
CCl_4-hepatotoxicity (1.25 ml/kg BW)	61.56 ± 5.15^{b}	0.36 ± 0.03^{b}	2.62 ± 0.18^{b}
CCl_4 + CRA (20 mg/kg BW)	$107.67 \pm 8.11^{a,c}$	0.72 ± 0.05^{c}	$5.48 \pm 0.42^{a,c}$

Values are expressed as means ± standard deviation (SD) for eight rats in each group. Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT).

decreased by CCl_4 administration and those pretreated with CRA above enzymic and non enzymic antioxidants were increased. CCl_4 not only initiates lipid peroxidation, but also reduces tissue SOD, CAT and GPx, activities, and this depletion may result from oxidative modification of these proteins (Augustyniak and Wazkilwicz, 2005).

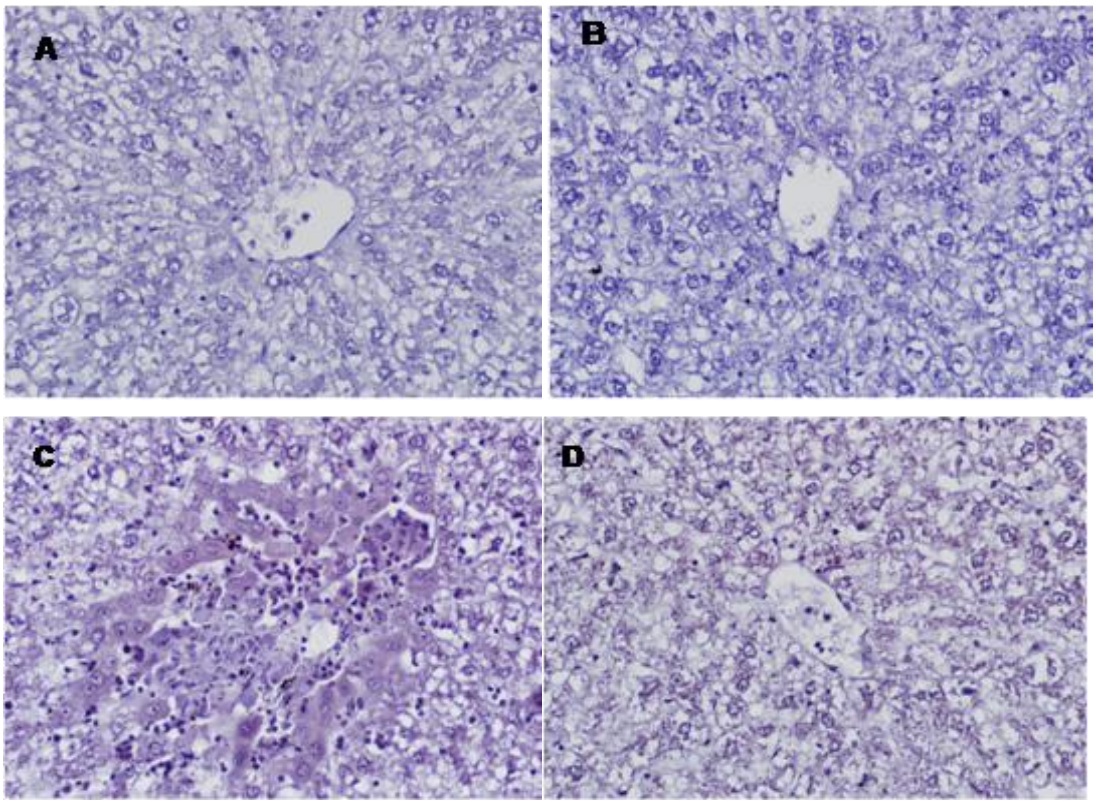

Figure 1. Histopathological changes of liver (H&E, 40×). (A) Control rats showing normal hepatocytes; (B) Control rats treated with CRA showing normal hepatocytes and central vein; (C) CCl_4-induced hepatotoxic rats showing degeneration of hepatocytes with nuclei pyknosis, increased vacuolation of cytoplasm and mononuclear cellular infiltration; (D) CCl_4-induced hepatotoxic rats treated with CRA showing normal hepatocytes and central vein.

Cells have a number of mechanisms to defend themselves from the toxic effect of ROS including free radical scavengers and chain reaction terminators such as SOD, CAT, and GPx systems. SOD removes superoxide radicals by converting them into H_2O_2 which can be rapidly converted into water by CAT and GPx. Cellular injury occurs when ROS generation exceeds the cellular capacity of removal (Wu et al., 2009). CRA administration effectively protected against the loss of these antioxidant activities after CCl_4 administration. Excessive liver damage and oxidative stress caused by CCl_4 depleted the levels of GSH, vitamin C and vitamin E in our study. Oxidative stress induced by CCl_4 results in the increased utilization of GSH and subsequently the levels of GSH is decreased in plasma and tissues. Utilization of vitamin E is increased when oxidative stress is induced by CCl_4 and this shows the protective role of vitamin E in mitigating the elevated oxidative stress. Vitamin C scavenges and destroys free radicals in combination with vitamin E and glutathione (George, 2003). It also functions cooperatively with vitamin E by regenerating tocopherol from the tocopheroxyl radical (Kaneto et al., 1999). A decrease in the levels of vitamin C may indicate increased oxidetive stress and free radical formation in CCl_4-induced liver injury. CRA treatment effectively restored the depleted levels of these non enzymic antioxidants. In the present study, the elevation of GSH levels in plasma and tissues was observed in the CRA treated rats. This indicates that the CRA can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH or have both effects. Increase in GSH levels could also contribute to the recycling of other antioxidants such as vitamin E and vitamin C (Exner et al., 2000). The histological changes in the liver of control and CCl_4-induced hepatotoxic rats are as shown in Figure 1. CCl_4-induced hepatotoxic rats showed degeneration of hepatocytes with pyknosis of their nuclei, increased vacuolation of their cytoplasm and some mononuclear cellular infiltration was also seen in and around the damaged areas. Treatment with coro-solic reduced these changes to near normalcy.

Conclusions

The results of this study demonstrate that CRA has a potent hepatoprotective action upon CCl_4-induced hepatic damage in rats. Our results show that the hepatoprotec-

tive and antioxidant effects of CRA may be due to its antioxidant and free radical scavenging properties. The biochemical findings were supported by histopathological study.

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