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Hepatoprotective effect of electrolyzed reduced water against carbon tetrachloride-induced liver damage in mice

Chia-Fang Tsai [a,1], Yu-Wen Hsu [a,1], Wen-Kang Chen [b], Wen-Huei Chang [c], Cheng-Chieh Yen [d], Yung-Chyuan Ho [c], Fung-Jou Lu [a,c,e,*]

[a] Institute of Medicine, College of Medicine, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung 402, Taiwan
[b] National Tainan Institute of Nursing, No. 78, Sec. 2, Minzu Rd., Tainan, Taiwan
[c] School of Applied Chemistry, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung 402, Taiwan
[d] School of Occupational Safety and Health, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung 402, Taiwan
[e] Department of Nutrition, Chung Shan Medical University Hospital, No. 110, Sec. 1, Jianguo N. Rd., Taichung 402, Taiwan

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ABSTRACT

The study investigated the protective effect of electrolyzed reduced water (ERW) against carbon tetrachloride (CCl_4)-induced liver damage. Male ICR mice were randomly divided into control, CCl_4, CCl_4 + silymarin, and CCl_4 + ERW groups. CCl_4-induced liver lesions include leukocytes infiltration, hepatocyte necrosis, ballooning degeneration, mitosis, calcification, fibrosis and an increase of serum alanine aminotransferase (ALT), and aminotransferase (AST) activity. In addition, CCl_4 also significantly decreased the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). By contrast, ERW or silymarin supplement significantly ameliorated the CCl_4-induced liver lesions, lowered the serum levels of hepatic enzyme markers (ALT and AST) and increased the activities of SOD, catalase, and GSH-Px in liver. Therefore, the results of this study show that ERW can be proposed to protect the liver against CCl_4-induced oxidative damage in mice, and the hepatoprotective effect might be correlated with its antioxidant and free radical scavenging effect.

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1. Introduction

Among the various health problems suffered by people in Taiwan, liver diseases including hepatocellular carcinoma, fibrosis, cirrhosis and hepatitis appear to be some of the most serious (Wu et al., 1999). Documented evidence suggested that reactive oxygen species (ROS) are known to play a crucial role in liver disease's pathology and progression (Vitaglione et al., 2004). ROS including superoxide and hydroxyl radicals have been proved to associate with the intoxication by carbon tetrachloride (CCl_4) (Slater and Sawyer, 1971). Experimentally induced cirrhotic response in animals by CCl_4 is shown to be superficially similar to human cirrhosis of the liver (Taira et al., 2004; Lee et al., 2007; Rudnicki et al., 2007). Thus, CCl_4-induced hepatic injury has been extensively used in the experimental models to evaluate the therapeutic potential of drugs and dietary antioxidants.

Many studies have suggested that natural antioxidants are efficacious to prevent oxidative stress-related liver pathologies due to particular interactions and synergisms (Bhathal et al., 1983; Vitaglione et al., 2004). A major defense mechanism involves the antioxidant enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px), which convert active oxygen molecules into non-toxic compounds. One of such candidates is electrolyzed reduced water (ERW), which was chosen in the present study.

ERW is generated by electrolysis of tap water and exhibits high pH, low oxidation–reduction potential, low dissolved oxygen, and high dissolved hydrogen. Shirahata et al. (1997) first described that ERW exhibits both SOD-like and catalase-like activities. They also found that the SOD-like activity of ERW is stable at 4 °C for over a month and is not lost even after neutralization, repeated freezing and melting, deflation with sonication, vigorous mixing, boiling, repeated filtration, or closed autoclaving. ERW has anticancer effects through inactivation of ERK and down-regulation of vascular endothelial growth factor (VEGF) expression in A549 cells (Ye et al., 2008). ERW inhibits tumor growth and intravenous metastasis of B16 melanoma in tumor-injected mice (Lee et al., 2004). ERW

Abbreviations: ALT, alanine aminotransferase; AST, aminotransferase; CCl_4, carbon tetrachloride; ERW, electrolyzed reduced water; GSH, glutathione; GSH-Px, glutathione peroxidase; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; SOD, superoxide dismutase; TG, triglyceride.

* Corresponding author. Address: Institute of Medicine, College of Medicine, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung 402, Taiwan. Tel.: +886 4 24730022x11872; fax: +886 4 23248189.
E-mail address: fjlu@csmu.edu.tw (F.-J. Lu).

[1] Both authors contributed equally to this work.

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protects pancreatic β cells from damage induced by alloxan, a diabetogenic agent (Li et al., 2002), increases the release of circulating insulin, and improves insulin sensitivity in diabetic animal models (Kim and Kim, 2006; Kim et al., 2007). Huang et al. (2003) suggested that ERW treatment in clinical trial is beneficial for lessen oxidative damage to leukocytes and endothelial cells in patients with renal diseases. Recently, we showed that ERW acts as an antioxidant and a potent ROS scavenger. Most importantly, when ERW was used in combination with GSH, an enhanced apoptosis-inducing effect on HL-60 cells was observed (Tsai et al., 2009). However, the effect of ERW on CCl_4-induced hepatotoxicity is still unclear.

In present study, we evaluated the potential hepatoprotective effects of ERW against CCl_4-induced hepatic damage in male ICR mice. Mice were orally treated with ERW or silymarin (as standard reference) daily accompany CCl_4 administration twice a week for 8 weeks. Hepatic GSH levels as well as activities of AST, ALT, cholesterol and TG in serum and catalase, SOD, and GSH-Px in liver were measured to monitor liver injury. The extent of CCl_4-induced liver injury was also analyzed through histopathological observations.

2. Materials and methods

2.1. Chemicals

Silymarin was obtained from the Sigma Chemical Co. All other chemicals and reagents used were obtained from local sources and were of analytical grade.

2.2. Apparatus of the ERW

Apparatus (Antioxidant Water System H.C., Health Control Co., Ltd, Taipei, Taiwan) for producing the ERW was continuous type and supplied commercially. The apparatus consisted of two parts. One was used for the purification of water while the other was used for the electrolysis of water. The equipment for the electrolysis of water would control the pH regulator from 8.10 to 10.1, ORP values from −160 mV to −607 mV, water flow rate from 2.0 L/min to 3.4 L/min, alkalinity, acidity, and purity of water. The entire apparatus was connected to a water tap. After opening the switch, the tap water was first purified and then electrolyzed to produce both the reduced water and oxidized water. The ERW was collected and used in this experiment.

2.3. Animals

Male ICR mice (20 ± 2 g) were obtained from the Animal Department of BioLASCO, Taiwan Company and were allowed to quarantine and acclimate for a week prior to experimentation. Mice were maintained on 12-h light/dark cycles in a temperature and humidity controlled room. Animals were allowed free access to food and water beside ERW group. Our Institutional Animal Care and Use Committee approved the protocols for the animal study, and the animals were cared for in accordance with the institutional ethical guideline.

2.4. Treatment

The animals were randomly divided into four groups with each consisting of 12 mice. The experimental groups were as follows. Group I served as normal control. Groups II–IV were administered orally 1 ml/kg body weight of CCl_4 (20% v/v in olive oil) twice a week for a period of 8 weeks. After CCl_4 intoxication, Group II served as control CCl_4. Group III served as positive control and was given silymarin (200 mg/kg, p.o.) daily for a period of 8 weeks. Group IV was received ERW everyday for a period of 8 weeks. At the end of the experiment, animals were sacrificed by cervical dislocation. Blood was collected into non-heparinized capillary tubes and centrifuged (1500 rpm, 10 min, 4 °C). Serum was aspirated and stored at −20 °C until assayed as described below. The liver was also removed and stored at −70 °C until use.

2.5. Measurement of serum ALT, AST, cholesterol, and TG

Serum ALT, AST, cholesterol, and TG were measured using commercial kits produced by Randox Laboratories Ltd. kit (UK) to assess the hepatotoxicity.

2.6. Measurement of SOD, catalase, GSH, and GSH-Px in liver homogenate

Liver was thawed, weighed and homogenized in Tris–HCl (5 mmol/L containing 2 mmol/L EDTA, pH 7.4). Homogenates were centrifuged (10,000 rpm, 10 min, 4 °C) and the supernatant was used immediately for the assays of SOD, catalase, GSH, and GSH-Px. All of these enzymes were determined following the instructions on the Randox Laboratories Ltd. kit.

2.7. Histopathological evaluation

The livers were preserved in 100 ml/L neutral buffered formalin solution and processed routinely by embedding in paraffin. Tissue sections (4–5 μm) were stained with hematoxylin and eosin (H&E) as well as Masson Trichrome and Gomor's reticulin silver stain and examined under light microscope for histopathological changes. The histological indices of hepatic inflammation, necrosis, bile duct proliferation, mitosis, and calcification were quantified based on Knodell et al's. (1981) method. The liver damage was graded as following: −, absent; +, few; ++, mild; and +++, moderate. Hepatic fibrosis was graded according to the method of Ruwart et al. (1989) as the following: −, absent, normal liver; +, few, increase of collagen without formation of septa; ++, mild, formation of incomplete septa from portal tract to central vein (septa that do not interconnect with each other); and +++, moderate, complete but thin septa interconnecting with each other (incomplete cirrhosis). The final numerical score was calculated by dividing the sum of the number per grade of affected mice by the total number of examined mice.

2.8. Statistical analysis

All values are expressed as means ± SD. Comparison between any two groups was performed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using computerized program. Statistically significant differences between groups were defined as $p < 0.05$.

3. Results

3.1. Effects of ERW on body weight and other organs

Table 1 shows that body weights of the experimental animals were not affected by CCl_4, silymarin, and ERW. However, a significant elevation of relative liver and spleen weight was seen in CCl_4-treated group, indicating that CCl_4 induced hypertrophy of these tissues. By contrast, silymarin or ERW in combination with CCl_4 significantly reduced the elevated weight of liver, suggesting the possibility of ERW to give protection against liver injury upon CCl_4 induction.

3.2. Effect of ERW in CCl_4-induced hepatotoxicity

The effect of ERW on ALT, AST, cholesterol, and TG are summarized in Table 2. Mice treated with CCl_4 showed higher serum level

Table 1
Effects of ERW and silymarin on body weight and relative organ weight in CCl_4-intoxicated mice.

Group	Body weight (g)	Relative weight (g/g body weight, %)		
		Heart	Liver	Spleen
Normal control	40.00 ± 2.95	0.46 ± 0.03	5.77 ± 0.34*	0.38 ± 0.05*
CCl_4 control	39.92 ± 3.15	0.49 ± 0.07	7.15 ± 0.44**	0.50 ± 0.14**
Silymarin (200 mg/kg) + CCl_4	41.33 ± 2.53	0.46 ± 0.07	6.19 ± 0.35*	0.40 ± 0.08
ERW + CCl_4	41.08 ± 3.48	0.46 ± 0.07	6.13 ± 0.47*	0.44 ± 0.08

All values are means ± SD ($n = 12$).
* $p < 0.05$ compared with CCl_4 group.
** $p < 0.05$ compared with normal group.

Table 2
Effects of ERW on serum ALT, AST, cholesterol, and TG in CCl_4-intoxicated mice.

Group	ALT (U/L)	AST (U/L)	Cholesterol (mg/dl)	TG (mg/dl)
Normal control	90.44 ± 18.09	55.85 ± 8.88	2.93 ± 0.44	1.06 ± 0.13
CCl_4 control	409.36 ± 74.83**	192.62 ± 36.64**	4.12 ± 0.53**	1.45 ± 0.19**
Silymarin (200 mg/kg) + CCl_4	129.47 ± 28.45*	95.83 ± 29.04*	2.91 ± 0.21*	1.14 ± 0.17*
ERW + CCl_4	129.47 ± 28.45*	72.99 ± 16.86*	2.96 ± 0.49*	1.05 ± 0.09*

All values are means ± SD ($n = 12$).
* $p < 0.05$ compared with CCl_4 group.
** $p < 0.05$ compared with normal group.

of ALT, AST, cholesterol, and TG as compared to normal controls ($p < 0.05$), indicating that CCl_4-induced hepatotoxicity. ERW administration during CCl_4 treatment caused a significantly reduction serum levels of ALT, AST, cholesterol, and TG in comparison with those observed in the CCl_4-treated group ($p < 0.05$). Positive control drug, silymarin, at dose of 200 mg/kg also reduced the level of serum enzymes. These results suggested the possibility of ERW to give protection against liver injury upon CCl_4 induction.

3.3. Hepatic antioxidant enzyme activities

The CCl_4-induced hepatotoxicity is mediated by primary and secondary bond formation of reactive species to critical cellular molecules such as DNA, lipid, proteins, or carbohydrates. Free radical scavenging enzymes and non-enzymatic antioxidant are important against the cell stress situation caused by CCl_4. Significantly decreased liver SOD and GSH-Px activities were observed in mice from the CCl_4-treated group as compared to the normal control group ($p < 0.05$). ERW administration as well as silymarin prevented the decrease due to CCl_4 and restored the activity of SOD and GSH-Px to almost normal. The catalase levels were no difference between the normal control group and the CCl_4-treated group. By contrast, administration with ERW and silymarin significantly increased the catalase levels as compared to the CCl_4-treated group (Table 3).

3.4. Histopathological evaluation

The CCl_4-induced histopathological changes in the liver with significant degeneration and necrosis of hepatocytes in the centrilobular region and with perivenular inflammatory infiltrates. Histopathologic examinations such as hepatocyte necrosis, inflammatory cell infiltration, ballooning degeneration, mitosis, calcification, and hepatocyte fibrosis were recorded and scored in Table 4. In normal control animals, liver sections showed normal hepatic cells with well preserved cytoplasm, prominent nucleus and nucleolus, and central vein (Fig. 1A). The liver sections of animals treated with CCl_4 showed a moderate degree of centrilobular necrosis, and mild degree of infiltration of leukocytes, bile duct proliferation, mitosis, and calcification (Fig. 1B). The histological observations also supported the results obtained from the serum enzyme assay. The histological pattern of the livers of the mice treated with ERW showed a few to milder degree of infiltration of leukocytes and absent to few degree of necrosis, and bile duct proliferation (Fig. 1D). Similar trends were also observed in animals treated with silymarin (Fig. 1C).

Further, histopathological changes of fibrosis occurred in CCl_4-intoxicated and prevention by the treatment with ERW are shown in Fig. 2. The livers of mice treated with CCl_4 showed extensive accumulation of connective tissue resulting in formation of continuous fibrotic septa, nodules of regeneration, and noticeable alterations in the central vein as compared to the normal control (Fig. 2A and B). The group intoxicated with CCl_4 and treated with ERW or silymarin resulted in less pronounced destruction of the liver architecture without fibrosis (Fig. 2C and D). According to microscopic examinations, severe hepatic fibrosis induced by CCl_4 was remarkably reduced by the administration of ERW, which was in good correlation with the results of the serum aminotransferase activities and hepatic antioxidant enzyme activities.

Table 3
Effects of ERW on liver SOD, catalase, GSH-Px, and GSH in CCl_4-intoxicated mice.

Group	SOD (Units/mg protein)	Catalase (Units/mg protein)	GSH-Px (nmole NADPH/min/mg protein)	GSH (μmol/g wet weight)
Normal control	407.63 ± 40.66	15.94 ± 2.07	337.17 ± 78.94	4.50 ± 0.45
CCl_4 control	255.98 ± 40.65**	13.53 ± 4.11	171.88 ± 21.11**	4.10 ± 0.63
Silymarin (200 mg/kg) + CCl_4	353.83 ± 61.52*	21.64 ± 2.42*	263.72 ± 29.08*	6.22 ± 0.48*
ERW + CCl_4	299.08 ± 28.84*	20.29 ± 3.50*	271.98 ± 30.01*	4.44 ± 1.18

All values are means ± SD ($n = 12$).
* $p < 0.05$ compared with CCl_4 group.
** $p < 0.05$ compared with normal group.

Table 4
Effects of ERW on hepatic histopathology of liver damage in mice treated with CCl_4.

Microscopic observation	Grades[a]	Normal control	CCl_4 control	Silymarin (200 mg/kg) + CCl_4	ERW + CCl_4
Infiltration of leukocytes (inflammation)	–	7	0	1	1
	+	5	2	10	7
	++	0	9	1	4
	+++	0	1	0	0
Hepatocyte necrosis	–	6	0	6	6
	+	6	4	5	6
	++	0	4	1	0
	+++	0	4	0	0
Bile duct proliferation	–	11	0	5	9
	+	1	1	4	3
	++	0	10	3	0
	+++	0	1	0	0
Mitosis	–	4	0	2	3
	+	7	3	9	9
	++	1	9	1	0
	+++	0	0	0	0
Calcification	–	12	3	7	10
	+	0	3	4	2
	++	0	6	3	0
	+++	0	0	1	0
Hepatocyte fibrosis[b]	–	12	0	7	9
	+	0	4	5	3
	++	0	8	0	0
	+++	0	0	0	0

[a] The histological indices of hepatic inflammation, necrosis, bile duct proliferation, mitosis, and calcification were quantified based on Knodell et al. (1981) method. The liver damage was graded as following: –, absent; +, few; ++, mild; and +++, moderate.

[b] For estimation of fibrosis, livers were sectioned and stained with Masson Trichrome and Gomor's reticulin silver stain by standard techniques. Hepatic fibrosis was graded according to the method of Ruwart et al. (1989) as the following: –, absent, normal liver; +, few, increase of collagen without formation of septa; ++, mild, formation of incomplete sẽpta from portal tract to central vein (septa that do not interconnect with each other); and +++, moderate, complete but thin septa interconnecting with each other (incomplete cirrhosis).

4. Discussion

ERW, produced by electrolysis of water near the cathode, exhibits high pH, low dissolved oxygen, extremely high dissolved molecular hydrogen, and extremely negative redox potential values (Shirahata et al., 1997; Kim and Kim, 2006). In the past year, molecular hydrogen dissolved in water has been reported to prevent or ameliorate diseases associated with oxidative stress in rodents and human. Yanagihara et al. (2005) demonstrated that use of the electrolyzed hydrogen-saturated water for drinking elicits an antioxidative activity in rats. Nagata et al. (2008) revealed that molecular hydrogen dissolved in drinking water prevents stress-induced learning impairment in mice. Ohsawa et al. (2008) showed that consumption of hydrogen water reduces atherosclerosis in apolipoprotein E knockout mice. Kajiyama et al. (2008) clarified that supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with Type 2 diabetes or impaires glucose tolerance. Nakashima-Kamimura et al. (in press) illustrated that molecular hydrogen alleviates cisplatin-induced

nephrotoxicity. Fu et al. (2009) presented that hydrogen water is likely able to retard the development and progression of Parkinson's disease in a rat model. Several studies also directly demonstrated that ERW has free radical scavenging activity and protects DNA, RNA, proteins, cells, and tissues against strong oxidative stress (Shirahata et al., 1997; Lee et al., 2006). A clinical study reported (Huang et al., 2006) that ERW treatment is effective in palliating hemodialysis-evoked oxidative stress in patients under chronic hemodialysis. Recently, we proved that ERW is an effective scavenger for H_2O_2, superoxide, and hydroxyl radical detected by the chemiluminescence as well as in a 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced cultured system (Tsai et al., 2009). Therefore, we considered that ERW is useful in preventing various hepatic damages induced by oxidative stress. In the present study, the capability of ERW to protect CCl_4-induced hepatotoxicity and oxidative stress was first investigated.

Carbon tetrachloride (CCl_4) is a xenobiotic that produces hepatotoxicity in various experimental animals. CCl_4 is metabolized by cytochrome P450 to form a reactive trichloromethyl radical (CCl_3) and a trichloromethyl peroxyl radical (CCl_3O_2). Both radicals are capable of binding to DNA, lipids, proteins or carbohydrates, leading to lipid peroxidation, cell necrosis, excessive deposition of collagen in liver, and liver fibrosis (Weber et al., 2003; Basu, 2003). Many studies have demonstrated that an important mechanism

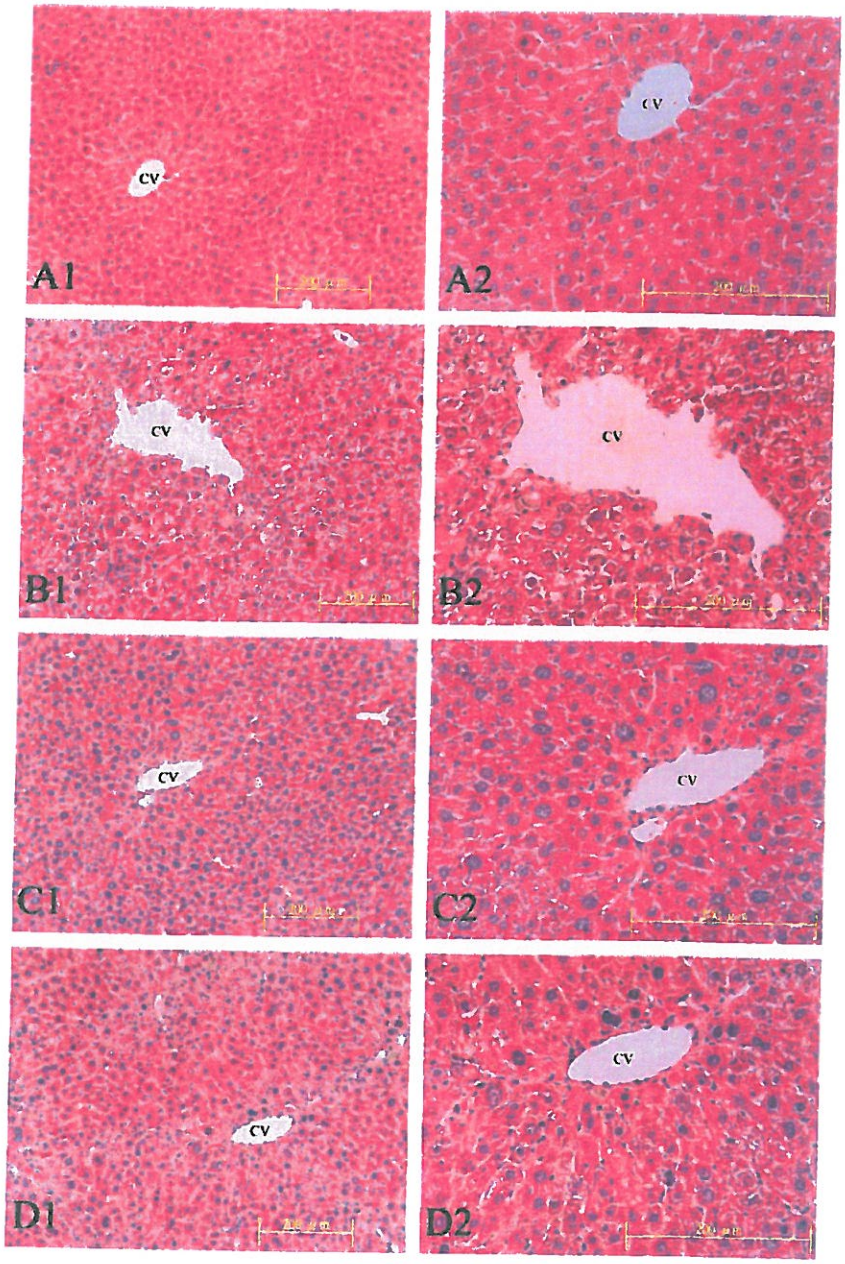


Fig. 1. Photomicrographs of liver sections stained with hematoxylin and eosin. (A) Hepatic tissue of control mice, showing normal appearance and central vein (CV). (B) Hepatic tissue of CCl_4-treated mice, showing moderate hepatocyte necrosis around the central vein region and mild inflammatory cell infiltration. (C) Hepatic tissue of silymarin (200 mg/kg) and CCl_4-treated mice, showing absent to few degree of hepatocyte necrosis and a few to milder degree of inflammatory cell infiltration. (D) Hepatic tissue of ERW and CCl_4-treated mice, showing absent to few degree of hepatocyte necrosis and a few to milder degree of inflammatory cell infiltration. Other details are provided in the text. Original magnification 200× (A1, B1, C1, and D1); 400× (A2, B2, C2, and D2).

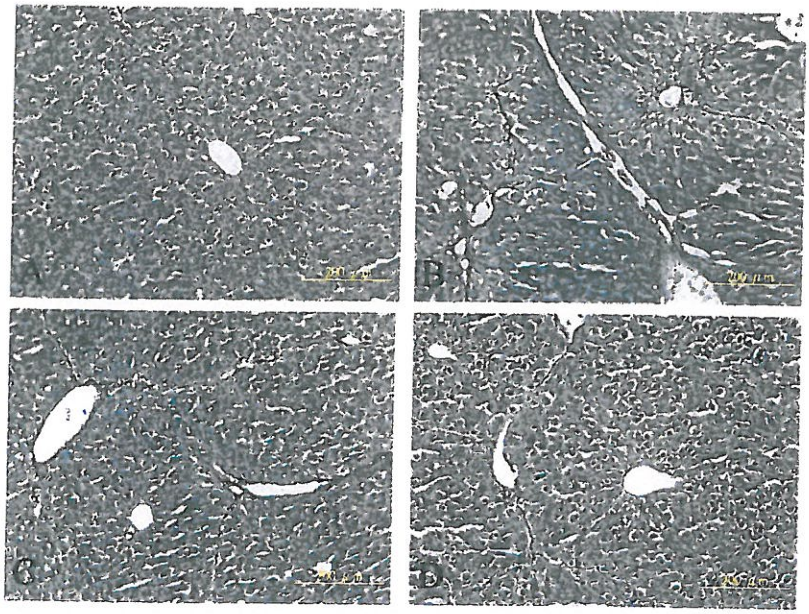


Fig. 2. Photomicrographs of liver sections stained with Masson Trichrome and Gomor's reticulin silver stain (200×). (A) Hepatic tissue of control mice, showing normal appearance. (B) Hepatic tissue of CCl_4-treated mice, showing moderate fibrosis, nodules of regeneration, and noticeable alterations in the central vein. (C) Hepatic tissue of silymarin (200 mg/kg) and CCl_4-treated mice, showing milder degree of hepatocyte fibrosis. (D) Hepatic tissue of ERW and CCl_4-treated mice, showing milder degree of hepatocyte fibrosis. Other details are provided in the text.

of the hepatoprotective effects may be related to an antioxidant capacity to scavenge reactive oxygen species (Hattori et al., 1991; Naik and Panda, 2007). Indeed, a considerable animal model of experimental has reported that several antioxidant agents, such as *Antrodia camphorata* extract (Hsiao et al., 2003), *Rhodiola sachalinensis* extract (Nan et al., 2003), caffeic acid phenethyl ester (Lee et al., 2008) and saponins derived from the roots of *Platycodon grandiflorum* (Lee et al., 2008) reduce CCl_4-induced hepatotoxicity by prevention of peroxidation. In the present study, we found that treatment with the ERW markedly inhibits CCl_4-induced liver damage as evidenced by decreased serum activities of AST and ALT, and reduced serum concentration of TG and cholesterol (Table 2). The biochemical observations are supported by the histopathological examination of the mice liver (Fig. 1).

SOD is an effective defense enzyme that catalyses the dismutation of superoxide anions into hydrogen peroxide (H_2O_2) (Reiter et al., 2000). Catalase is a haemeprotein in all aerobic cells that catalyses the H_2O_2 to oxygen and water and protects the tissue exemption from oxidative damage by highly reactive oxygen free radicals and hydroxyl radicals. GSH-Px is an important enzyme in the detoxification of xenobiotics in the liver and converted the reduction of H_2O_2 and hydroperoxides to non-toxic products (Baudrimont et al., 1997; Naik and Panda, 2007). Lipid peroxides or ROS easily inactivate these antioxidant enzymes, which results in reduced activities of these enzymes in CCl_4 toxicity (Yang et al., 2008). GSH is an extremely efficient intracellular buffer for oxidative stress and GSH acts as a non-enzymatic antioxidant that reduces H_2O_2, hydroperoxides (ROOH) and xenobiotic toxicity (Kadiska et al., 2000). In the present study, the hepatic antioxidant enzymatic of SOD and GSH-Px activities were significantly decreased in CCl_4-intoxicated mice compared with control mice, implying increased oxidative damage to the liver. On the contrary, SOD, catalase, and GSH-Px levels were significantly elevated by administration of ERW to CCl_4-intoxicated mice, suggesting that it has the ability to restore these enzymes' activities in CCl_4-damaged liver. However, administration of ERW to CCl_4-intoxicated mice was no different in hepatic content of GSH compared to the CCl_4-treated group, suggesting that ERW markedly inhibits CCl_4-induced liver damage by elevated hepatic antioxidant enzymatic system such as SOD, catalase, and GSH-Px.

In conclusion, the results of this study demonstrate that the ERW was effective in prevention of CCl_4-induced hepatic damage in mice. Our results show that the hepatoprotective effects of ERW may be due to increase of antioxidant enzymes activity. This is the first to report the hepatoprotective effects of ERW in *vivo*. According to the results of the present study, the ERW possess a potent antioxidant activity. Since oxidation is known to be involved in the pathogenesis of many diseases, treatment with ERW is claimed to be effective. The inhibitory effects of a dietary ERW may be useful as a hepatoprotective agent against chemical-induced hepatotoxicity *in vivo*. Further studies should be continued to carry out in order to assess the role and elucidate the action mechanism.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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