Food Chemistry 151 (2014) 148–153

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Hepatoprotective effects of fermented *Curcuma longa* L. on carbon tetrachloride-induced oxidative stress in rats

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ARTICLE INFO

Article history:
Received 10 June 2013
Received in revised form 8 November 2013
Accepted 11 November 2013
Available online 19 November 2013

Keywords:
Fermented *Curcuma longa* L.
Hepatoprotection
Oxidative stress
Antioxidation
Carbon tetrachloride

ABSTRACT

The hepatoprotective effect of fermented *Curcuma longa* L. (FC) was investigated in rats under CCl_4-induced oxidative stress. FC at a dose of 30 or 300 mg/kg body weight (b.w.) was orally administered for 14 days followed by a single dose of CCl_4 (1.25 mL/kg b.w. in 20% corn oil) on day 14. Pretreatment with FC drastically prevented the elevated activities of serum AST, ALT, LDH, and ALP caused by CCl_4-induced hepatotoxicity. Histopathologically evident hepatic necrosis was significantly ameliorated by FC pretreatment. When compared to the CCl_4-alone treated group, rats pretreated with FC displayed the reduced level of malondialdehyde. Furthermore, FC enhanced antioxidant capacities with higher activities of catalase, glutathione-*S*-transferase, glutathione reductase, and glutathione peroxidase, and level of reduced glutathione. These results suggest that FC could be a candidate used for the prevention against various liver diseases induced by oxidative stress *via* elevating antioxidative potentials and decreasing lipid peroxidation.

1. Introduction

Curcuma longa L., known as turmeric, is a perennial herb that is widely cultivated in tropical regions of Asia and Africa. It has been used for a long time in many Asian countries as a traditional medicine for wound healing, inflammation, and stomach acidity (Ammon & Wahl, 1991). Some studies suggested the efficacy of *C. longa* L. as anti-atherosclerotic, anti-diabetic, anti-mutagenic, anti-cancer, and antioxidant agents (Huang et al., 1994; Nishiyama et al., 2005). *C. longa* L. is also used for the food applications as an active ingredient in curries and mustards (Srinivasan, 2005). However, its strong flavour and taste decrease consumer palatability and limit the industrial application, which requires improvement of its characterisation. Fermented *C. longa* L. (FC) has properties including reduced bitterness and harsh taste that increase consumer acceptance (Kang et al., 2009).

Due to its unique metabolism and relationship to the gastrointestinal tract, the liver is an important target of the toxicity of drugs and xenobiotics (Jaeschke et al., 2002). In recent years, natural agents with improved effectiveness and safety profiles as a therapy for liver disease have been extensively sought because the administration of drugs sometimes meets with limited therapeutic success and is associated with serious complications, especially for the long-term use (Bishayee, Darvesh, Politis, & McGory, 2010; Jaishree & Badami, 2010).

An imbalance between endogenous antioxidant defence system and reactive oxygen species (ROS) level in the body causes oxidative stress, which is associated with overproduction of free radicals from various liver disorders, such as alcoholic liver damage, non-alcoholic fatty liver disease, and drug-induced liver injury (Lu et al., 2013; Sumida, Niki, Naito, & Yoshikawa, 2013; You et al., 2009). ROS derived from many sources influence macromolecules in the liver, resulting in hepatotoxicity (Lu & Cederbaum, 2008). In general, ROS can be scavenged by detoxifying system within the body, such as glutathione, glutathione peroxidase, and catalase (Mohammadi & Yazdanparast, 2009). However, quantities of ROS

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http://dx.doi.org/10.1016/j.foodchem.2013.11.058

that overwhelm the capacity of the body's defence system lead to a disturbance of homoeostases in ROS production and antioxidant defence, which in turn eventually damages biological molecules and key cellular components and processes such as lipid peroxidation, enzyme inactivation, and oxidative DNA damage (Albano, 2008). Dietary antioxidant supplementation may help to restore this balance, eventually inhibiting the hepatotoxicity (Chen, Li, Lin, Chien, & Low, 2011; Tatiya, Surana, Sutar, & Gamit, 2012). Therefore, plant products or alternative medicines that could limit ROS-mediated injuries are needed to aid in the protection of the liver from possible damage.

In the present study, *C. longa* L., which had been microbially fermented and developed to improve flavour and taste, was investigated for its *in vivo* protective effects on liver damage induced by carbon tetrachloride (CCl_4). Also, the mode of action relevant to the *C. longa* L.'s antioxidative activity in liver damage was determined. The knowledge gained might aid in the development of effective therapeutics on liver damage.

2. Materials and methods

2.1. Sample and chemicals

C. longa L. was authenticated by Dr. Humyoung Baek at Korea INS Pharm Research Institute (Hwasun, Korea). A voucher specimen was deposited at the same institute. The FC was standardise and supplied by Korea INS Research Institute. Raw *C. longa* L. was harvested and obtained from Jindo, Jellanamdo, Korea. *C. longa* L. was prepared as a 200 mesh powder by grinding and sieving. The powder was fermented by 2% *Aspergillus oryzae* followed by sterilization at 121 °C for 15 min and lyophilized. Catalase (CAT), glutathione-*S*-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), reduced glutathione (GSH), and CCl_4 were purchased from Sigma–Aldrich (St. Louis, MO, USA). All chemicals were of analytical reagent grade.

2.2. Animals

Eight-week-old male Sprague–Dawley rats with a body weight (b.w.) of 183.0 ± 18.9 g were purchased from Orient Bio (Seongnam, Korea) and housed in cages under automatically controlled air conditions of temperature (22 ± 2 °C), humidity (about 60%), and lighting (12:12-h light–dark cycle). The rats were fed a commercial pelleted chow (AIN-76A rodent purified diet, Orient Bio) and water *ad libitum*. The Institutional Animal Care and Use Committee of Chonnam National University approved the protocol for the animal study, and the animals were cared for in accordance with the "Guidelines for Animal Experiments" established by the university.

2.3. Experimental groups

The rats were acclimatised for one week prior to use in experiment 1 or 2.

Experiment 1: For the test of toxic effect of FC, 24 healthy male rats were divided into four groups ($n = 6$ per group). Group 1 rats (control group) received the respective vehicles only. Group 2, 3, and 4 rats were administered 30, 100, and 300 mg/kg b.w./day of FC dissolved in corn oil, respectively. Each group received the appropriate vehicle or sample daily by gastric intubation for 14 days. Body weights were recorded in the beginning and at the end of the experiment. Clinical signs were observed daily. Two weeks after the initial treatment, the rats were sacrificed 12 h after final FC treatment under light ether anaesthesia to collect blood and tissues. These were stored at −70 °C.

Experiment 2: To evaluate the protective effect against CCl_4-induced hepatotoxicity, 24 rats were divided into four groups ($n = 6$ per group). Two groups were administered FC by gastric intubation for 14 consecutive days at doses of 30 or 300 mg/kg b.w./day. The other two groups similarly received distilled water and served as a normal control and a CCl_4 control. At day 14, normal control rats were given 20% corn oil and the other rats were administered an acute oral dose of CCl_4 (1.25 mL/kg b.w. in 20% corn oil) 3 h after final administration. Rats were sacrificed 12 h after CCl_4 treatment under light ether anaesthesia in order to collect blood and liver. These were stored at −70 °C.

2.4. Assay for serum marker enzymes

Sera were used for the spectrophotometric determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) using commercially available diagnostic kits supplied by Asanpharm (Seoul, Korea).

2.5. Assay for hepatic antioxidant activities

For the antioxidant activity assays, liver tissue was homogenated in 50 mM phosphate buffer. The resulting suspension was then centrifuged at 13,000*g* for 15 min at 4 °C, and the supernatant was used for the measurement. The activity of CAT was measured according to the method of Aebi (1984). Hepatic GST activity was determined as described by the method of Habig and Jakoby (1981). GR activity was assayed using an adaptation of Carlberg and Mannervik's method (1975). GPx activity was monitored according to the method of Paglia and Valentine (1967). The level of GSH was estimated by the method of Akerboom and Sies (1981). The concentration of malondialdehyde (MDA) was assayed by monitoring thiobarbituric acid reactive substance formation as described by Draper and Hadley (1990). The amount of protein was measured using the Bradford assay (1976).

2.6. Histopathological examinations

For the histopathological analysis, a portion of the median lobe of the liver was dissected and fixed in 10% buffered formalin solution for 24 h. After routine processing, the liver tissues ($n = 6$ per group) were embedded in paraffin, sectioned at a thickness of 5 μm, and stained with haematoxylin and eosin (H & E) for photomicroscopic assessment. All observations were made manually with a light microscope with 5×, 10×, 20×, and 40× objective lenses and a 100× oil immersion lens in a totally blinded manner. The following variables were used for assessment of histological changes of the liver: (1) hepatocyte degeneration/necrosis; (2) fatty changes; (3) inflammatory cell infiltration; and (4) congestion.

2.7. Statistical analysis

Data are presented as mean ± S.D. of 6 replicates. The data were statistically evaluated using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to compare significant differences between the groups at $p < 0.05$.

3. Results

3.1. Safety of fermented C. longa L. on rats

The oral toxicity study with FC was performed at doses of up to 300 mg/kg b.w./day. During the test period, all FC-administered

rats survived and none of the treated rats displayed toxic symptoms (Table 1). Also, 14 days of FC treatment up to 300 mg/kg b.w./day did not significantly affect body weight gain. The levels of serum ALT and AST, which are established reliable markers for liver function, revealed no significant changes in any rat administered FC. Dosages of 30 and 300 mg/kg b.w./day were chosen as the non-toxic level to carry out the subsequent study.

3.2. Effects of fermented C. longa L. on CCl_4-induced hepatotoxicity

The activities of ALT, AST, LDH, and ALP were significantly elevated at 12 h following CCl_4 administration to rat, as evidenced in Table 2. However, FC pretreatment for 14 days significantly decreased the levels of the serum biochemical indicators for the CCl_4-induced hepatic damages. Notably, administration of FC at the dose of 30 mg/kg b.w./day recovered the impaired liver functions resulting from CCl_4-induced toxicity.

3.3. Changes in antioxidant enzyme activities by fermented C. longa L. on CCl_4-induced hepatotoxicity

The functions of liver antioxidant enzymes by the induction of CCl_4 to rat are summarise in Table 3. In the CCl_4 dosed group, CAT activity was decreased by approximately 19% when compared to the normal control group. However, pretreatment with FC for 14 days from the dose of 30 mg/kg b.w./day completely prevented a decrease in CAT activity. A dose-dependent manner was found in CAT activity between low (30 mg/kg b.w./day) and high (300 mg/kg b.w./day) FC administered groups. Consistent with the CAT activity, the administration of FC significantly protected against the CCl_4-induced depletion of GST activity, except for no significant change between low and high doses. The GR activities in two FC-pretreated groups also showed the statistically significant enhancement in comparison to the CCl_4 dosed group. Hepatic GPx activity declined by 17% in the CCl_4 dosed group compared to the normal control group, while no reduction was significant in rats that received FC.

3.4. Effects of fermented C. longa L. on GSH and MDA levels

Changes in GSH level on CCl_4 dosed rats pretreated with FC are depicted in Fig. 1. Here, the oxidative stress induced by CCl_4 caused a significant decrease in the level of GSH, a key intracellular non-enzymatic antioxidant, in comparison with the normal control group (9.96 ± 0.70 *vs.* 14.02 ± 0.16 μmole/mg protein, respectively). On the other hand, the pretreatment of FC at 30 mg/kg b.w./day partially prevented the reduction of GSH level, and that at 300 mg/kg b.w./day almost completely prevented the depletion of GSH. As shown in Fig. 2, the concentration of MDA, an end point product of lipid peroxidation, in the CCl_4-treated rats was higher by 33% than that in the normal control group. However, pretreatment with FC lead to a decrease in MDA level compared to the CCl_4 dosed group.

Table 1
Effects of 14 day exposure to fermented *Curcuma longa* L. on weight, survival and hepatic markers in the serum of rats.*

Group (mg/kg b.w./day)	Weight gain (g)	AST** (Karmen)	ALT** (Karmen)
0	$27.1 \pm 1.0^{a,***}$	179 ± 6^{a}	37.2 ± 3.9^{a}
30	28.1 ± 1.2^{a}	173 ± 7^{a}	39.7 ± 4.7^{a}
100	30.7 ± 2.8^{a}	162 ± 7^{a}	34.4 ± 2.8^{a}
300	30.2 ± 1.6^{a}	168 ± 5^{a}	39.3 ± 3.5^{a}

* Data express the mean ± S.D. for 6 rats.
** AST, aspartate aminotransferase; ALT, alanine aminotransferase.
*** Values with different letters in a column are significantly different by one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

3.5. Effects of fermented C. longa L. on histopathological morphology

In normal control rats, no abnormal appearance or histopathological change were observed in the liver. When compared to the normal liver tissues, CCl_4 administration caused hepatic damage in rat liver, as demonstrated by severe heptatocyte necrosis, inflammatory cell infiltration, haemorrhage, and hepatic lobular disorganisation (Fig. 3). However, histopathological hepatic lesions induced by CCl_4 administration were ameliorated by the pretreatment of FC.

4. Discussion

FC was similarly safe to *C. longa* L. as no significant toxicity was noted after high dose of FC was orally provided to rats. According to the previous study, no significant toxic effects were observed in rats given 500 mg/kg b.w./day of *C. longa* L. (Hallagan, Allen, & Borzelleca, 1995).

Hepatocytic damage is characterised by hepatic marker enzymes including ALT, AST, ALP, and LDH. When liver cells are damaged, these enzymes leak into the bloodstream from liver tissue and produce markedly elevated serum levels (Kasdallah-Grissa et al., 2007). Our results provide evidence that CCl_4 administration causes severe acute liver damage in rats. These adverse effects of CCl_4 on liver function are in line with previous studies (Domitrovic, Jakovac, Milin, & Radosevic-Stasic, 2009; Huo, Wang, Liang, Bao, & Gu, 2011). The reduction in activities of these biochemical parameters by FC pretreatment is an indication of prevention of hepatic tissue damage caused by CCl_4. These results were further confirmed by histopathological examinations. The acute hepatotoxicity by CCl_4 administration revealed hepatocellular necrosis and apoptosis, fatty accumulation, inflammatory cell infiltration, and other histological manifestations, consistent with previous findings (Domitrovic et al., 2009; Jia et al., 2011). According to the microscopic examination of FC-pretreated rat liver cells during acute intoxication of CCl_4, the incidence and severity of histopathological hepatic lesions were significantly prevented, which correlated well with the results of serum enzymes.

The present study was undertaken to evaluate the hepatoprotective effects of FC on CCl_4-induced oxidative stress in liver of Sprague–Dawley rats. CCl_4 is a hepatotoxin that has been used extensively to induce liver injury in various experimental models to elucidate the mechanisms underlying hepatotoxicity (Marques et al., 2012). Of the various mechanisms involved in the hepatotoxic effect of CCl_4, one is oxidative damage through free radical generation (Higuchi & Gores, 2003). CCl_4-mediated hepatotoxicity is developed from the biotransformation of CCl_4 by cytochrome P450 2E1 to the trichloromethyl free radical ($^{\cdot}CCl_3$). This free radical is further converted to a highly reactive species ($CCl_3O_2^{\cdot}$) by the reaction with oxygen. Trichloromethylperoxy radical covalently bonds to cellular macromolecules and leads to a chain reaction of polyunsaturated fatty acids in the cytoplasmic membrane phospholipids, causing functional and morphological changes to the cell membrane, and finally, cell necrosis (Weber, Boll, & Stampfl, 2003). Thus, the inhibition of excessive ROS production might be an important mechanism involved in the protection against CCl_4-induced liver damage. MDA is widely used as a marker of lipid peroxidation and a major parameter for the status of oxidative stress (You et al., 2010). Balasubramaniyan, Sailaja, and Nalini (2003) reported that the hepatic MDA level was increased under the enhancement of oxidative stress in a rodent model. Our study revealed that rats treated with CCl_4 exhibited a significant increase

Table 2
Effects of fermented *Curcuma longa* L. on hepatic markers in the serum of CCl_4-treated rats.*

Group	AST** (Karmen)	ALT** (Karmen)	LDH** (Wrohlewski)	ALP** (K-A)
Normal control	178 ± 5[a,****]	30.9 ± 1.2[a]	1788 ± 70[a]	22.0 ± 1.2[a]
CCl_4 control	289 ± 4[b]	112.7 ± 1.6[b]	2652 ± 105[b]	43.6 ± 2.6[b]
FCL***	272 ± 3[c]	75.9 ± 6.2[c]	2099 ± 33[c]	33.5 ± 0.1[c]
FCH***	251 ± 3[d]	62.1 ± 6.4[d]	2066 ± 41[c]	29.6 ± 2.2[d]

* Data express the mean ± S.D. for 6 rats.
** AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.
*** FCL: Pretreated with 30 mg/kg b.w. of fermented *Curcuma longa* L. plus CCl_4, FCH: Pretreated with 300 mg/kg b.w. of fermented *Curcuma longa* L. plus CCl_4.
**** Values with different letters in a column are significantly different by one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

Table 3
Changes in enzymatic antioxidant activities by the administration of fermented *Curcuma longa* L. to CCl_4-treated rats.*

Group	CAT** (U/mg protein)	GST** (U/mg protein)	GR** (U/mg protein)	GPx** (U/mg protein)
Normal control	2.73 ± 0.06[a,****]	37.8 ± 0.5[a]	2.99 ± 0.05[a]	6.06 ± 0.09[a]
CCl_4 control	2.20 ± 0.05[b]	29.1 ± 0.6[b]	2.73 ± 0.05[b]	5.02 ± 0.21[b]
FCL***	2.41 ± 0.01[c]	33.1 ± 0.9[c]	3.20 ± 0.08[a]	5.81 ± 0.11[a]
FCH***	2.85 ± 0.06[a]	34.4 ± 0.9[c]	3.13 ± 0.03[a]	5.88 ± 0.09[a]

* Data express the mean ± S.D. for 6 rats.
** CAT, Catalase; GST, Glutathione-*S*-transferase; GR, Glutathione reductase; GPx, Glutathione peroxidase.
*** FCL: Pretreated with 30 mg/kg b.w. of fermented *Curcuma longa* L. plus CCl_4, FCH: Pretreated with 300 mg/kg b.w. of fermented *Curcuma longa* L. plus CCl_4.
**** Values with different letters in a column are significantly different by one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

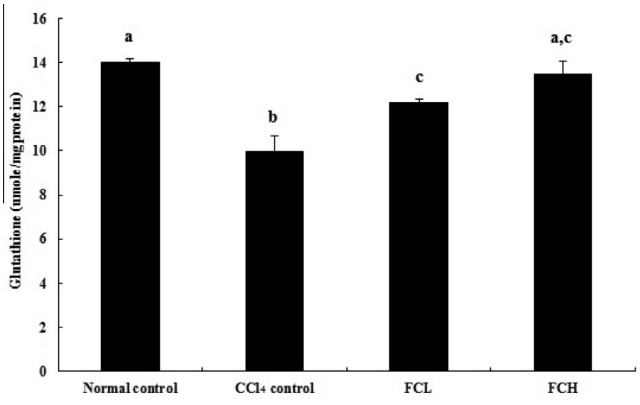

Fig. 1. Effects of fermented *Curcuma longa* L. on hepatic glutathione level in CCl_4-treated rats. Data express the mean ± S.D. for 6 rats. Different letters above the bar indicate statistically significant differences by one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$). FCL: Pretreated with 30 mg/kg b.w. of fermented *Curcuma longa* L. plus CCl_4, FCH: Pretreated with 300 mg/kg b.w. of fermented *Curcuma longa* L. plus CCl_4.

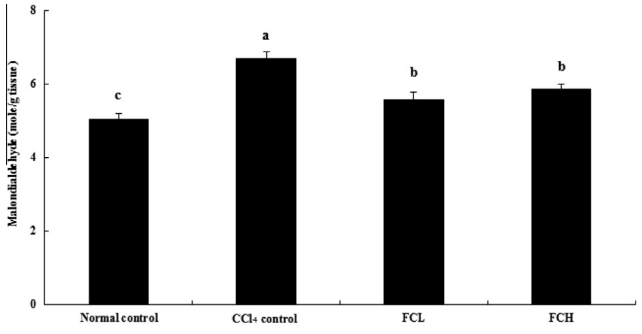

Fig. 2. Effects of fermented *Curcuma longa* L. on hepatic malondialdehyde level in CCl_4-treated rats. Data express the mean ± S.D. for 6 rats. Different letters above the bar indicate statistically significant differences by one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$). FCL: Pretreated with 30 mg/kg b.w. of fermented *Curcuma longa* L. plus CCl_4, FCH: Pretreated with 300 mg/kg b.w. of fermented *Curcuma longa* L. plus CCl_4.

in MDA level compared to the control group. Pretreatment with FC significantly reduced the CCl_4-induced hepatic MDA elevation, implying that FC could provide protective effects against CCl_4-induced liver damage in terms of preventing lipid peroxide formation and blocking oxidative chain reaction.

Overproduced ROS could directly attack the hepatocellular membrane as a result of lipid peroxidation, followed by a cascading series of cellular events such as the massive release of inflammatory cytokines, which ultimately lead to liver injuries (Higuchi & Gores, 2003; Wu & Cederbaum, 2009). Administration of CCl_4 elevates the formation of lipid peroxides and ROS, leading to the inactivation of enzymatic and non-enzymatic antioxidants in the liver. Therefore, it is valuable to identify natural antioxidants that can antagonise the deleterious action of excessive ROS to protect hepatocytes from damage. As expected, the capacities of hepatic antioxidants including GSH, CAT, GST, GPx, and GR were declined by CCl_4 administration to rats, which is in agreement with earlier studies (Shahjahan, Sabitha, Jainu, & Devi, 2004). The defence system in the body also includes small molecules such as GSH and vitamin E as well as antioxidant enzymes (Mohammadi & Yazdanparast, 2009). Hepatic GSH is an important regulator of intracellular redox homoeostases, which plays a crucial role in scavenging ROS and maintaining antioxidant enzymes. In the present study, depletion of GSH obtained by CCl_4 exposure may reflect its rapid utilisation by the overproduction of ROS and subsequent oxidative stress. Pretreatment with FC helped to eliminate ROS and relieve oxidative stress induced by CCl_4, as evidenced by preventing the depletion of hepatic GSH level.

Endogenous antioxidant enzymes such as CAT, GST, GPx, and GR serve as the first-line defence against ROS and in the maintenance of cellular redox balance. However, excessive ROS production by CCl_4-induced oxidative stress may lead to the exhaustion of endogenous antioxidant defence mechanisms, resulting in inactivation of antioxidant enzymes. Decreased levels of CAT, GST, GPx, and GR in rats following the administration of CCl_4 suggest hepatic damage, largely due to an overwhelming oxidative modification of the enzymatic proteins by increased ROS production. Our observations revealed that FC supplementation ameliorated the impaired antioxidative defence system in rat livers, as indicated by the restoration of enzyme activities.

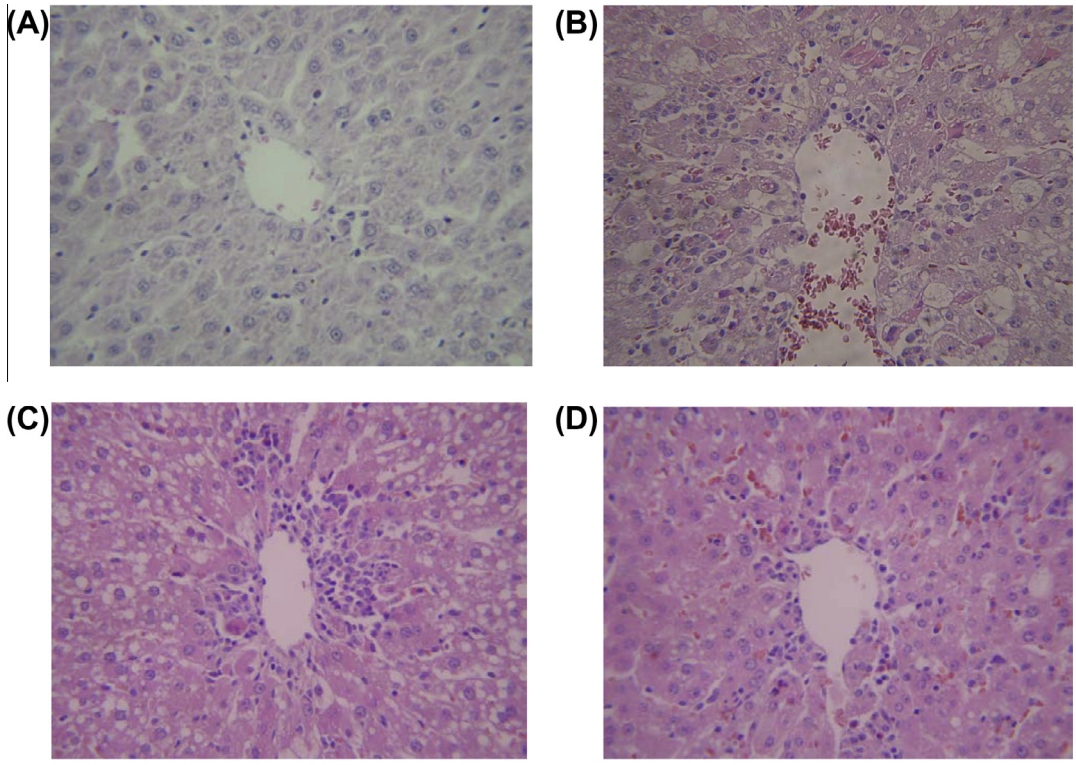

Fig. 3. Effects of fermented *Curcuma longa* L. on liver histopathological change in CCl_4-treated rats. The liver sections were stained with haematoxylin and eosin (H & E) to demonstrate the histopathological morphology (400×). (A) Normal control; (B) CCl_4 control; (C) FCL, pretreated with 30 mg/kg b.w. of fermented *Curcuma longa* L. plus CCl_4; (D) FCH, pretreated with 300 mg/kg b.w. of fermented *Curcuma longa* L. plus CCl_4.

In conclusion, the present study is the first to report that pretreatment of fermented *C. longa* L. is effective in the prevention of CCl_4-induced hepatic damage in rats. It is presumed that the mechanism of hepatoprotection by FC supplementation against CCl_4 toxicity might be due to the alleviation of oxidative stress *via* preventing lipid peroxidation and ameliorating hepatic antioxidant status. The development of dietary supplementation using fermented *C. longa* L. could be useful to protect against liver damage mediated by oxidative stress.

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