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The Protective Effects of *Curcuma longa* Linn. Extract on Carbon Tetrachloride-Induced Hepatotoxicity in Rats *via* Upregulation of Nrf2

Lee, Hyeong-Seon[1], Li Li[1], Hyun-Kyung Kim[1,2], Dinesh Bilehal[1], Wei Li[1], Dong-Seok Lee[1], and Yong-Ho Kim[1]*

[1]*Department of Smart Foods and Drugs, Inje University, Gimhae 621-749, Korea*
[2]*Department of Biomedical Laboratory Science, Gimcheon University, Gimcheon 740-704, Korea*

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This study was designed to investigate the potentially protective effects of *Curcuma longa* Linn. extract (CLE) on carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. Male Sprague-Dawley rats were pretreated with 50 or 100 mg/kg of CLE or 100 mg/kg of butylated hydroxytoluene (BHT) for 14 days before CCl_4 administration. In addition, the CLE control group was pretreated with 100 mg/kg CLE for only 14 days. Three hours after the final treatment, a single dose of CCl_4 (20 mg/kg) was administrated intraperitoneally to each group. After the completion of this phase of the experiment, food and water were removed 12 h prior to the next step. The rats were then anesthetized by urethane and their blood and liver were collected. It was observed that the aspartate aminotransferase and alanine aminotransferase activities of the serum, and the hepatic malondialdehyde levels had significantly decreased in the CLE group when compared with the CCl_4-treated group. The antioxidant activities, such as superoxide dismutase, catalase, and glutathione peroxidase activities, in addition to glutathione content, had increased considerably in the CLE group compared with the CCl_4-treated group. Phase II detoxifying enzymes, such as glutathione S-transferase, were found to have significantly increased in the CLE group as opposed to the CCl_4-treated group. The content of Nrf2 was determined by Western blot analysis. Pretreated CLE increased the level of nuclear translocated Nrf2, and the Nrf2 then increased the activity of the antioxidant and phase II detoxifying enzymes. These results indicate that CLE has protective effects against CCl_4-induced hepatotoxicity in rats, *via* activities of antioxidant and phase II detoxifying enzymes, and through the activation of nuclear translocated Nrf2.

**Corresponding author*
Phone: +82-55-320-3481; Fax: +82-55-334-3426;
E-mail: mlskimyh@inje.ac.kr

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The liver is a vital organ that plays a key role in the detoxification of endogenous and exogenous substances secreted in the body. In addition, the liver eliminates harmful substances in xenobiotic-induced hepatotoxicity [2, 48].

Carbon tetrachloride (CCl_4) is a potent toxin that is commonly used to induce hepatotoxicity in experimental models; a single dose of CCl_4 can rapidly lead to the production of free radicals and thus induce acute liver damage [2, 48, 49]. A hepatic cytochrome P450 (CYP450)-mediated biotransformation of CCl_4 leads to the production of hepatotoxic metabolites, trichloromethyl free radicals ($CCl_3^{\cdot}$) and/or trichloromethyl peroxyl radicals ($CCl_3OO^{\cdot}$). Trichloromethyl free radicals can covalently bind to cellular molecules (nucleic acids, proteins, and lipids) and lead to membrane lipid peroxidation [24], which ultimately leads to apoptosis and necrosis [15, 27]. It has been reported that CYP450 in rat liver activates CCl_4 and stimulates Kupffer cells [28] to produce reactive oxygen species (ROS), such as $^{\cdot}O_2^-$, H_2O_2, and $^{\cdot}OH$, which damage the liver [27, 36]. These ROS are eliminated by antioxidants and phase II detoxifying enzymes such as glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST) [25, 39]. Upregulation of many phase II detoxifying and antioxidant enzymes is mediated by antioxidant response elements (ARE). The transcription factor nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) plays a pivotal role in the activation of ARE-driven antioxidant gene expression [6]. Nrf2 is a member of the "cap 'n' collar"

family of basic leucine zipper (bZIP) transcription factors [42]. Of these, Nrf2 is expressed in many tissues such as that of the liver, kidneys, skin, and lungs [6, 31]. Nrf2 in an inactive state is sequestered by its suppressor Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. Upon cell stimulation, Nrf2 translocates into the nucleus where it binds to the ARE and leads to the expression of target genes [9, 42].

A yellow-colored pigment is found in turmeric, or *Curcuma longa* Linn. (Zingiberaceae), a perennial herb distributed mainly throughout tropical and subtropical regions. India is the leading producer of *Curcuma longa* Linn., but it is also cultivated in Bangladesh, China and its surrounding islands, Indonesia, the islands of the Caribbean, and in several South American countries [43]. Turmeric is usually used as a dietary pigment and spice. Yellow pigments such as curcumin, *p*-coumaroyl feruloyl methane, di-*p*-coumaroyl methane, and other pigments are present in turmeric [5]. Furthermore, turmeric has been used traditionally as a herbal medicine for the treatment of a number of diseases [19, 30].

Turmeric has potent antiviral [20], antimutagenic [38], anti-inflammatory [13], and antioxidant [7] properties and is even used in the treatment of injuries to the skin [41]. However, the mechanisms underlying the hepatoprotective, and other chemoprotective effects elicited by *Curcuma long*a Linn. extract (CLE), in association with Nrf2 upregulation, remain poorly understood. In the present study, we investigated whether CLE pretreatment exerted protective effects on CCl_4-induced hepatotoxicity in rats. In addition, we attempt to elucidate the possible mechanisms underlying any such protective effect.

Materials and Methods

Materials and Chemicals

A powder of *Curcuma longa* Linn. was purchased from the local Korean market. Carbon tetrachloride (CCl_4), butylated hydroxytoluene (BHT), and corn oil were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The glutathione (GSH) assay kit, catalase (CAT) assay kit, superoxide dismutase (SOD) assay kit, glutathione peroxidase (GPx) assay kit, and glutathione S-transferase (GST) assay kit were acquired from the Cayman Chemical Co. (Michigan, U.S.A.). The lipid peroxidase assay kit was purchased from Oxford Biomedical Research, Inc. (Oxford, MI, U.S.A.). A polyclonal rabbit antibody against Nrf2 was purchased from Santa Cruz Biochemistry, Inc. (CA, U.S.A.). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloroindolyphosphate (BCIP) were purchased from Promega Corporation (Madison, WI, U.S.A.). All other reagents were of analytic grade.

Extraction of *Curcuma longa* Linn.

Curcuma longa Linn. powder was purchased from a local market and stored under refrigerated conditions. Turmeric powder (500 g) was put through the extraction process, in batches of 50 g, through the use of a Soxhlet apparatus for 24 h, using absolute ethanol as the solvent (150 ml in each case). The extracts were then cooled to room temperature and filtered using Whatman filter paper No. 1. The ethanol filtrate was concentrated using a Heidolph rotary evaporator (Laborota 4000, Heidolph, Germany) through to dryness. In total, 32.5 g of *Curcuma longa* Linn. crude oil was extracted from 500 g of *Curcuma longa* Linn. powder.

GC−MS Analysis

All extracts were analyzed on a GC−MS system, which included a Shimadzu 2010 series (Shimadzu, Kyoto, Japan) of GC equipped with a AOC-20S automatic liquid sampler (Shimadzu, Kyoto, Japan) and interfaced directly to a QP2010 mass selective detector controlled *via* the accompanying data system. A 30 m DB-5MS capillary column (0.25 mm id, 0.25 μm film) was used. The temperature program was as follows: from 30 to 150°C at 20°C/min and then 150−280°C at 10°C/min. The injection volume was 1 μl in a splitless mode. Helium was used as the carrier gas with a 1 ml/min flow rate. The temperature of the injector was maintained at 220°C. A solvent delay of 5 min was maintained throughout. Tuning was performed using the autotune feature with perfluorotributylamine (PFTBA); the electron multiplier voltage was nominally kept at 1,500 V. All data were obtained by collecting the full-scan mass spectra within the scan range of 350−550 amu. The GC−MS interface line and MS inlet temperatures were 280 and 240°C, respectively, and the ion-source temperature was 280°C.

Animals and Treatment

Five-week-old male Sprague-Dawley (SD) rats (130−140 g) were purchased from Hyo-Chang Science Co. (Daegu, Korea). The animals were nurtured with Purina Rodent Chow and tap water. They were maintained under standard conditions at a temperature of 22±2°C, and a relative humidity of 60±5%, with a 12 h light/dark cycle, and acclimatized for 1 week before the experiment. The rats were randomized into six treatment groups (each group with at least six animals). Group I (Control) and Group II (CCl_4) rats received corn oil intragluteally (i.g.). Group III rats received BHT (100 mg/kg, i.g.), and Groups IV and V rats received CLE (50, 100 mg/kg, i.g.). Group VI rats received CLE only (100 mg/kg i.g.) every day for 2 weeks. Three hours after the final treatment, CCl_4 dissolved in corn oil (20 mg/kg) was administrated intraperitoneally (i.p.) to each group except Groups I and VI. After the completion of the experiment, food and water were removed 12 h prior to sacrifice. The rats were anesthetized by urethane, and blood was collected from the hepatic portal vein. The blood samples were allowed to clot at room temperature for 30 min and then centrifuged at 3,000 ×*g* for 15 min. The livers were quickly excised from the rats and whole livers were washed with normal cold saline (pH 7.4). The washed livers were then stored at −80°C until required for further use in the experiment. All experimental procedures were approved by the Inje University Animal Care and Use Committee (IUACUC) and met all the guidelines in the Care and Use of Animals.

Serum Analysis

The serum activities of AST and ALT were measured to assess hepatotoxicity, and the enzymatic activities of AST and ALT in seru were determined through an auto analyzer (200FR, TOSIBA, Tokyo, Japan).

Liver Homogenate Preparation

The liver from each rat was homogenized in 9 ml of a cold 50 mM potassium phosphate buffer (pH 7.4, containing 1 mM EDTA) per gram of tissue weight. The homogenates were centrifuged at 10,000 ×*g* for 15 min at 4°C and the supernatants were then removed for assay of CAT, GST activity, and GSH content. Samples for the SOD and GPx activity assays were prepared from liver tissue homogenization with 9 ml of a cold 20 mM HEPES buffer (pH 7.2) and a 50 mM Tris-HCl buffer (pH 7.5) per gram of tissue, respectively. Tissue homogenates were prepared with 9 ml of a cold 20 mM Tris buffer (pH 7.4) to which 10 µl of 0.5 M BHT stock solution was added per 1 ml of tissue homogenate in order to prevent sample oxidization. After homogenization, samples were centrifuged at 3,000 ×*g* for 10 min at 4°C and then removed from the supernatants for the determination of MDA levels. Total protein concentrations were measured by Bradford's method [4] using bovine serum albumin (Santa Cruz Biochemistry, Inc. U.S.A.) as the standard.

Determination of MDA Levels

Lipid peroxidation was measured by the reaction of a chromogen, *N*-methyl-2-phenylindole, with MDA. Measurements of MDA were used as indicators of lipid peroxidation [21]. One molecule of MDA reacts with 2 molecules of *N*-methyl-2-phenylindole to yield a stable chromophore.

A 140 µl aliquot of sample solution and MDA standard solution were added to each microcentrifuge tube, and 455 µl of diluted *N*-methyl-2-phenylindole was then added to each tube and vortexed. Then 105 µl of 37% HCl was added to each tube and mixed. Each microcentrifuge tube was incubated at 45°C for 60 min and centrifuged at 15,000 ×*g* for 10 min. Then 150 µl of the supernatant was transferred to a microplate and the absorbance measured at 586 nm using a fluorescence multidetection reader (Synergy HT, Biotek, U.S.A.). The level of MDA was expressed as uM MDA/mg homogenate protein.

Determination of Antioxidant Enzymatic Activity

SOD activity was determined by using a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to express a 50% dismutation of the superoxide radicals. The plate absorbance was measured at 450 nm using a fluorescence multidetection reader (Synergy HT, Biotek, U.S.A.). The activity of SOD was expressed as units per milligram of homogenate protein. CAT activity was measured by using the peroxidatic function of catalase. Determination of CAT activity was based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen [18, 47]. Absorbance of all wells was measured at 540 nm. The activity of CAT was expressed as nM CAT/mg homogenate protein.

GPx activity was measured by means of a reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon the reduction of hydroperoxide by GPx, was then recycled to its reduced state by GR and NADPH [32]. The oxidation of NADPH to $NADP^+$ was accompanied by a decrease in absorbance at 340 nm. The rate of decrease was directly proportional to the GPx activity in the sample. The absorbance in the wells was kinetically measured at 340 nm at 1-min intervals for 5 min. The activity of GPx was expressed as nM GPx/mg homogenate protein.

Determination of GSH Content

The content of GSH was determined through a kinetic method. The sulfhydryl group of GSH reacted with 5,5'-dithio-*bis*-2-(nitrobenzoic acid) (DTNB) to produce a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The yellow product's (GSTNB) absorbance was measured at 412 nm [3]. The absorbance in the wells was kinetically measured at 412 nm at 5-min intervals for 30 min. The content of GSH was expressed as µM GSH/mg homogenate protein.

Determination of GST Activity

GST activity was measured by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione [14, 21]. The conjugation was accompanied by an increase in absorbance at 340 nm. The rate of increase was directly proportional to the increases in GST activity in the sample. The absorbance in the wells was kinetically measured at 340 nm at 1-min intervals for 5 min. The activity of GST was expressed as nM GST/mg homogenate protein.

Preparation of Nuclear Fractions from Liver

The nuclear extract from the liver was prepared as follows: The tissue was homogenized in 10 volumes of an isotonic buffer (pH 7.4, 10 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40) and centrifuged at 14,000 ×*g* for 15 min at 4°C. The supernatant was then removed as a cytosolic fraction. The pellet was resuspended in a radioimmune precipitation assay buffer (RIPA) (pH 8.0, 50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 µM sodium fluoride, 1 mM sodium orthovanadate, and 1 µM okadaic acid) and centrifuged at 14,800 ×*g* for 10 min at 4°C. The supernatant was used as the nuclear fraction and stored at −80°C after determination of protein concentrations [9, 33, 42].

Western Blot Analysis of Nrf2

Nuclear fractions containing 30 µg of proteins were run on 10% sodium dodecyl sulfate–polyacrylamide gels for electrophoresis (Mini Format 1-D Electrophoresis Systems, Bio-Rad, Hercules, CA, U.S.A.). After electrophoresis, the gel was transferred to a PVDF membrane. The membrane was blocked for 16 h with 5% BSA buffer. After blotting, the membrane was incubated for 2 h at 4°C with a 1:500 dilution of primary antibodies (rabbit polyclonal against the Nrf2 antibody). The membrane was washed three times with a TBST buffer at 10-min intervals. After washing, the membrane was incubated for 2 h at 4°C with a 1:1,000 dilution of secondary antibodies (alkaline phosphates-conjugated goat anti-rabbit IgG) and again washed in TBST three times. Blots were detected using a mixture of 5-bromo-4-chloroindolyphosphate (BCIP) and nitroblue tetrazolium (NBT). The intensity of the bands was measured by densitometry analysis with PDQuest software (version 7.0, Bio-Rad. U.S.A.).

Statistical Analysis

All experiments were performed in triplicate. The results are represented as mean±SD. All experimental data were treated by Student's *t*-test analysis. A $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

It has been reported that CLE exhibits a number of beneficial properties making it potentially highly useful for

Table 1. Composition of *Curcuma longa* Linn. extract.

Retention time (min)	Component	Percentage of compositon
8.333	α-Farnesene	0.41
8.583	*ar*-Curcumene	4.38
8.758	Alloaromadendrene	2.73
8.917	β-Farnesene	10.61
9.158	*Trans*-α-bergamotene	0.21
9.342	d-Nerolidol	0.47
9.492	Germacrene B	0.64
9.750	β-Caryophyllene	1.42
9.833	Germacrone	1.43
10.033	α-Bisabolol	2.39
10.167	γ-Elemene	1.11
10.217	zingiberenol	1.85
10.475	*ar*-Tumerone	4.50
10.722	Caryophylene oxide	11.53
10.925	Curlone	9.59
11.150	Ledene	0.35
12.108	Tumerone	0.83
12.167	Camilol	0.56
12.908	δ-Guaiene	1.32
14.483	Verrucarol	0.77
17.517	Emersol	1.03

the treatment of a wide number and variety of diseases. Traditionally, CLE has been used to improve a range of physiological health problems, although its exact properties remain incompletely defined to modern science [43]. In the present study, CLE was analyzed using GC−MS, with its compound identifications being reported in Table 1. In addition, through this study, it has been demonstrated that CLE protects against CCl_4-induced hepatotoxicity in rats *via* the upregulation of Nrf2.

The rats were pretreated with BHT and CLE for 14 days in order to evaluate their protective effects against toxic agents. Induction of liver injuries by CCl_4 is the best characterized system of xenobiotic-induced hepatotoxicity, and is commonly used in the screening of hepatoprotective activity [23], and this method was employed for this study. Changes in the Nrf2 content, and in the activity of phase II detoxifying and antioxidant enzymes, were then examined.

Effects of CLE on CCl_4-Induced Hepatotoxicity

Serum activities of AST and ALT are the most commonly used biochemical markers of liver injury. Damaged liver tissue leads to the leakage of large quantities of these enzymes into the blood stream [10, 12]. The effects of CLE on AST and ALT activities in rats with CCl_4-induced hepatotoxicity are presented in Table 2. In this study, AST and ALT activities increased ($p<0.05$) in the group that was treated with CCl_4 alone, indicating damage to the liver tissue by CCl_4-induced hepatotoxicity. However, AST and ALT activities in the pretreated BHT and CLE groups were lower than that of the group not pretreated. This finding suggests that BHT and CLE work to protect liver tissue from injury. These results confirm those of previous reports [7, 29].

Effects of CLE on Hepatic MDA Levels

The metabolism of CCl_4 initiates lipid peroxidation in cell membranes. Peroxidation of both saturated and unsaturated lipids results in the production of unstable hydroperoxides. Such polyunsaturated fatty acids produce 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) [17, 21, 35]. The production of MDA is used as a biomarker to measure the level of oxidative damage in tissues [37, 45]. As shown in Table 2, the hepatic level of MDA was assessed as an indicator of lipid peroxidation. In the CCl_4-treated group, the MDA level significantly increased ($p<0.05$) when compared with the control group. The MDA levels in the BHT and CLE groups were lower than that of the solely CCl_4-treated group. The MDA level in the CLE (100 mg/kg) only group decreased approximately 1.8-fold as compared with the CCl_4-treated group. These findings indicate that CLE prevents the production of adducts by lipid peroxidation. This result demonstrates that CLE might have a hepatoprotective effect against CCl_4 intoxication.

Table 2. Effects of CLE on the activities of serum AST and ALT, contents of MDA and GSH, and activity of GST in rats.

Groups	AST (U/l)	ALT (U/l)	MDA (μM/mg protein)	GSH (μM/mg protein)	GST (nM/mg protein)
I	118.25±13.30*	44.25±2.50*	2.74±1.61*	18.89±2.10**	4.71±0.57*
II	189.33±30.09$^{\#}$	81.33±17.95$^{\#}$	5.61±0.83$^{\#}$	12.40±1.62$^{\#\#}$	2.90±0.74$^{\#}$
III	118.67±18.97*	39.00±5.70*	2.63±1.34***	23.01±3.11**	6.12±0.82**
IV	156.00±16.87*	51.80±13.55*	4.23±0.43*	16.62±0.90**	4.24±0.67*
V	118.50±13.91*	37.80±6.38*	3.32±0.46**	22.80±3.99**	6.35±0.27$^{\#\#}$**
VI	130.00±23.43*	32.50±4.36$^{\#}$*	3.04±0.94**	27.72±0.76$^{\#\#\#}$***	7.02±0.16$^{\#\#}$**

The results are presented as a mean±SD of six animals in each group.

Group I: normal control; Group II: CCl_4 (20 mg/kg)-treated; Group III: BHT (100 mg/kg)+CCl_4; Group IV: CLE (50 mg/kg)+CCl_4; Group V: CLE (100 mg/kg) +CCl_4; Group VI: CLE (100 mg/kg) solely.

#, ##, ### Significantly different from Group I at $p<0.05$, $p<0.01$, $p<0.001$.

*, **, *** Significantly different from Group II at $p<0.05$, $p<0.01$, $p<0.001$.

Effects of CLE on Hepatic SOD Activity, CAT Activity, and GPx Activity

Overproduction of reactive cytotoxic molecules, caused by CCl_4 metabolism, can be suppressed by enzymatic and molecular antioxidants such as SOD, CAT, GPx, GST, and GSH [8, 46].

SOD catalyzes the dismutation of superoxide anions ($^{\cdot}O_2^-$) to hydrogen peroxide (H_2O_2) and oxygen (O_2), and thus forms a crucial part of cellular antioxidant defense mechanisms [1, 16, 40, 46]. As Fig. 1A shows, hepatic SOD activity was significantly lower ($p<0.05$) in the CCl_4-treated group than in the control group. SOD activity in both the BHT and CLE groups significantly increased compared with the CCl_4-treated group. In particular, the solely CLE (100 mg/kg)-treated group exhibited a significant increase of about 1.5-fold *vis-à-vis* the CCl_4 treated-group.

CAT is an antioxidant enzyme that is involved in the detoxification of hydrogen peroxide and reactive oxygen species. This enzyme catalyzes the conversion of hydrogen peroxide to oxygen and water [32, 47]. Hepatic CAT activity is shown in Fig. 1B. The CAT activity in the CCl_4-treated group was decreased about 1.5-fold compared with the control group. Both the BHT and CLE groups showed substantial increases *vis-à-vis* the CCl_4-treated group. In particular, the solely CLE (100 mg/kg) group displayed a very significant increase ($p<0.001$).

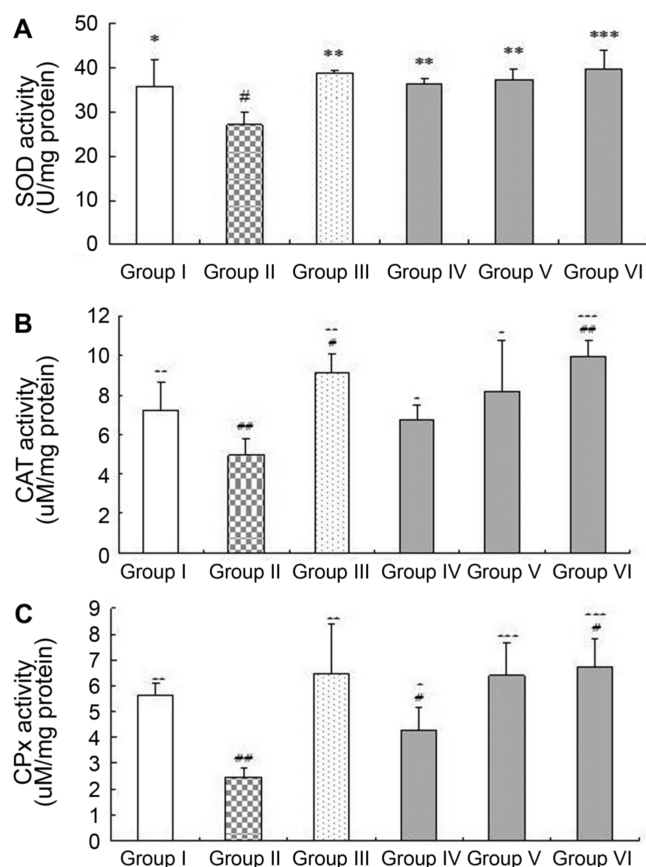

Fig. 1. Effects of CLE on hepatic SOD activity (**A**), CAT activity (**B**), and GPx activity (**C**) in rats.

The results are presented as a mean±SD of six animals in each group. Group I: normal control; Group II: CCl_4 (20 mg/kg)-treated; Group III: BHT (100 mg/kg)+CCl_4; Group IV: CLE (50 mg/kg)+CCl_4; Group V: CLE (100 mg/kg)+CCl_4; Group VI: CLE (100 mg/kg) solely. #, ## Significantly different from Group I at $p<0.05$, $p<0.01$. *, **, *** Significantly different from Group II at $p<0.05$, $p<0.01$, $p<0.001$.

GPx also catalyzes the reduction of hydrogen peroxide by reducing glutathione functions, thereby protecting the tissue from oxidative damage [18, 40]. Hepatic GPx activity is shown in Fig. 1C. The GPx activity in the CCl_4-treated group was significantly decreased ($p<0.01$) compared with that of the control group. Groups pretreated with CLE and BHT for 14 days exhibited substantial increases in the GPx activity *vis-à-vis* the CCl_4-treated group. The BHT and CLE groups displayed two times more GPx activity than the CCl_4-treated group, with the exception of the CLE 50 mg/kg group. Therefore, it can be surmised that antioxidant enzymes are easily inactivated by lipid peroxides or reactive oxygen species, which results in a decreased activity for these enzymes in CCl_4 toxicity.

In previous studies, decreased activity for SOD, CAT and GPx occurred in liver injury induced by CCl_4, implying a downregulation for numerous enzymatic oxidation reactions in the cell [21, 39, 44]. It has been suggested that the effect of antioxidants is influenced and decreased by the metabolism of CCl_4. Our study showed that the activities of SOD, CAT, and GPx in the CCl_4-treated group were significantly decreased ($p<0.05$) *vis-à-vis* the control group. The activities of SOD, CAT, and GPx in the BHT and CLE groups increased significantly compared with the CCl_4-treated group. This result demonstrates that CLE increases the antioxidant activities of these enzymes and protects the liver against CCl_4 intoxication. Therefore, an increase in the activities of these antioxidant enzymes may decrease the concentrations of ROS (*e.g.*, O_2^-, H_2O_2, and OH) produced by toxic agent metabolites.

Effects of CLE on Hepatic GSH Content and GST Activity

GSH is a tripeptide (γ-glutamylcysteinylglycine) that is widely distributed in a variety of tissues. The sulfhydryl (thiol) group (SH) of cysteine serves as a proton donor and is responsible for the biological activity of glutathione. GSH, an antioxidant, helps protect cells from ROS. GSH removes H_2O_2 and reacts with the trichloromethyl free radical from CYP450-mediated CCl_4 metabolites [26, 34]. The hepatic content of GSH is presented in Table 2. The GSH content in the CCl_4-treated group was substantially decreased ($p<0.01$) compared with that of the control group. Groups pretreated with CLE and BHT for 14 days, exhibited significant increases in GSH content *vis-à-vis* the CCl_4-treated group. The rats treated with CLE (100 mg/kg) from the CCl_4 group showed increases of about 1.8-fold

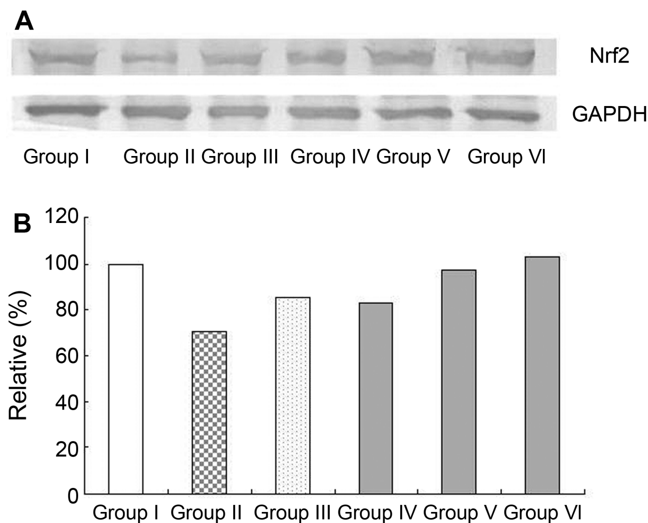

Fig. 2. Western blot analysis of hepatic Nrf2 content in rats (**A**) and densitometry analysis of the Western blot (**B**).
Group I: normal control; Group II: CCl_4 (20 mg/kg)-treated; Group III: BHT (100 mg/kg)+CCl_4; Group IV: CLE (50 mg/kg)+CCl_4; Group V: CLE (100 mg/kg)+CCl_4; Group VI: CLE (100 mg/kg) solely.

compared with the CCl_4-treated group. The content in the solely CLE (100 mg/kg) group was 2 times more than in the CCl_4-treated group.

GST is a phase II detoxifying enzyme that plays an important role in cellular detoxification and the excretion of xenobiotics. This enzyme protects cells against toxicants by conjugating them to GSH, thereby neutralizing their electrophilic sites and rendering the products more water-soluble [14]. GSH is a cofactor for GST, the latter being an abundant cellular enzyme in the tissue. The GSH conjugates are further metabolized to mercapturic acid and then excreted. Some compounds that increase the activity of GST, which metabolizes toxic compounds into non-toxic ones, have been found to protect the liver [26]. GST activity is presented in Table 2. The GST activity in the CCl_4-treated group was decreased about 40% *vis-à-vis* the control group. However, in the BHT and CLE groups, the GST activity was significantly increased compared with the CCl_4-treated group. The GST activity in the CLE groups increased two times more than in the CCl_4-treated group, with the exception of the CLE (50 mg/kg) group.

The hepatic GST activity and GSH content were markedly decreased by CCl_4 intoxication. The GST activity and GSH content in the CLE group were significantly increased compared with the CCl_4-treated group. This result shows that the protective effects of CLE against CCl_4-induced hepatotoxicity may be related to an increase in cellular GSH content, or an increase in GST activity.

Effects of CLE on Hepatic Nrf2 Content

Nrf2 plays a key role in the activation of antioxidants and phase II detoxifying enzymes by regulating their transcription [22]. Under normal conditions, Nrf2 is located in the cytoplasm where it forms an inactive complex with its repressor Kelch-like ECH-associated protein 1 [31, 39, 42]. Upon cell stimulation, Nrf2 dissociates from Keap 1 and translocates into the nucleus where it binds to ARE [9, 11, 33]. Several other previous studies have reported that antioxidant and phase II detoxifying enzymes are induced through upregulation of Nrf2 [9, 33, 39].

In this study, we found that CLE administration induced the nuclear translocation of Nrf2. The change in Nrf2 content was then investigated. Nrf2 content is displayed in Fig. 2A and 2B. In Western blot analysis, a marked decrease in hepatic Nrf2 protein expression was found in the CCl_4-treated group. Nrf2 protein expressions in the BHT and CLE (50 mg/kg) groups were slightly increased *vis-à-vis* the CCl_4-treated group. The CLE (100 mg/kg) group exhibited marked increases when compared with the CCl_4-treated group. These results suggest that CLE treatment induces an augmentation of hepatic Nrf2 protein levels and stimulates the activity of antioxidants and phase II detoxifying enzymes.

In conclusion, the results of this study demonstrate that CLE has a potent hepatoprotective function against CCl_4-induced hepatic injuries in rats. The increase in Nrf2 concentrations caused by CLE and its activation are consistent with a high activity of antioxidants and phase II detoxifying enzymes. The increase in Nrf2 content by CLE thus increases the induction of antioxidants and phase II detoxifying enzymes. In addition, the increase in the activity of antioxidants and phase II detoxifying enzymes by CLE decreases the concentration of ROS (*e.g.*, O_2^-, H_2O_2, and OH) caused by CCl_4 metabolites. Therefore this study demonstrates that CLE has a protective effect against CCl_4-induced hepatotoxicity in rats as a result of antioxidants and phase II detoxifying enzymes being activated by an upregulation of Nrf2.

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