Research Paper

Hepatoprotective Effect of *Houttuynia cordata* Thunb Extract against Carbon Tetrachloride-induced Hepatic Damage in Mice

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Houttuynia cordata Thunb (_Saururaceae_) is a traditional medicinal herb used to treat several disease symptoms. The present study was focused on the hepatoprotective effects of _H. cordata_ ethyl acetate extract in experimental mice. Further the antioxidant potential of the extract was also evaluated to substantiate its hepatoprotective properties. Carbon tetrachloride-induced hepatic damage in mice was used to measure the serum biochemical parameters. Morphological changes in hepatocyte architecture were studied by haematoxylin and eosin staining. _In vitro_ alkyl and hydroxyl free radical scavenging assays were performed to evaluate the antioxidant effect. Administration of _H. cordata_ extract significantly reduced the elevated serum levels and regulated the altered levels of serum cholesterol in carbon tetrachloride-treated mice ($P<0.05$). The morphological changes in hepatocyte architecture were also reversed by _H. cordata_ treatment. Further, the extract showed significant antioxidant actions by scavenging the alkyl and hydroxyl free radicals. The concentration of the extract necessary for 50% scavenging of alkyl and hydroxyl radicals was 15.5 and 410 µg/ml, respectively. _H. cordata_ extract exhibited significant hepatoprotective property in carbon tetrachloride-induced hepatotoxicity in mice. The strong antioxidant activities possessed by the extract might be responsible for such actions.

Key words: Heartleaf, antioxidant, hydroxyl radicals, hepatotoxicity, hyperlipidemia, carbon tetrachloride

Liver is an important organ which plays a central role in metabolic homeostasis[1]. Hepatitis, environmental pollutants, bacterial and viral infections, alcohol abuse are said to be some of the major factors leading to liver failure. In recent decades, the pharmaceutical application potential of natural products from herbs and dietary supplement has attracted much interest from researchers in treating liver complications[2,3]. In experimental hepatopathy, carbon tetrachloride (CCl_4) has been commonly used because it initiates oxidative damage, decreases the activities of antioxidant enzymes and generates toxic free radicals[4,5]. CCl_4-induced hepatotoxicity in animal models has been shown to be similar to human liver cirrhosis[6,7].

Antioxidants of plant origin have been demonstrated to either inhibit or prevent the progression of cellular disturbances resulting from the CCl_4-induced liver injury[8]. *Houttuynia cordata* Thunb (Saururaceae, *H. cordata*) is a perennial herb that is native to Southeast Asia. It is known as *Eo-Sung-Cho* in Korea and elaborates a wide spectrum of medicinal properties including allergic inflammation and anaphylaxis[9,10]. Traditionally, *H. cordata* was used as folk remedy for diuresis, antiviral, antibacterial and antileukemic activities[11]. However, the hepatoprotective protective actions and effect on serum lipid profiles of *H. cordata* in CCl_4-induced hepatotoxicity in experimental animals have not been addressed. In this study, *H. cordata* ethyl acetate extract was evaluated for its hepatoprotective effects using CCl_4-induced acute hepatic damage in experimental mice. Further the *in vitro* antioxidant activities of *H. cordata* extract has also been investigated.

One of the major features of metabolic syndrome is dyslipidemia, characterized by elevated levels of very low-density lipoprotein (VLDL), triglyceride (TG) and low density lipoprotein (LDL)[12]. Elevated plasma VLDL and TG levels are due, in large part, to an increase in hepatic overproduction of

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TG-enriched VLDL particles[13]. CCl_4-induced toxicity, which is thoroughly studied for its hepatotoxic properties, also in turn increases the accumulation of fat in the liver leading to hyperlipidemia. Mounting evidence suggests that reactive oxygen species (ROS) are known to play a pivotal role in liver-disease pathology and ROS have been proven to be associated with CCl_4-induced hepatotoxicity[14,15]. Therefore, antioxidant therapy might be an ideal approach to ameliorate liver damage caused by hepatotoxin.

MATERIALS AND METHODS

Male ICR mice (15-16 g) were obtained from NARA Biotech (Seoul, Korea). The animals were kept under standard laboratory conditions. A 12 h light/dark cycle in a temperature and humidity controlled room with water and food *ad libitum* was maintained. All animal experiments were performed in accordance and approval with our Institutional Animal Care and Use Committee of Konkuk University and the International Guidelines for Handling of Laboratory Animals[16]. Mice were anaesthetized by ketamine (160 mg/kg)/xylazine (40 mg/kg) cocktail injections, intraperitoneally.

Preparation of the *H. cordata* extract:
Dried plant material of *H. cordata* was purchased from the traditional herb market and was authenticated by a taxonomist at Konkuk University, South Korea. A voucher specimen (HC-KU2013) was kept in our department herbarium for future reference. To obtain the *H. cordata* extract, the dried plant material was ground in a mixer and defatted three times with three volumes of ethanol. The residue was extracted with absolute ethanol at 1:10 ratio (w/v) for 2 h in heating mantle at 70~80°. The supernatant was filtered and concentrated in a rotary evaporator at 50°. For further fractionation, the alcoholic extract (50 g) was partitioned into hexane, ethyl acetate (EA), and n-butanol fractions to yield 0.53, 8.72 and 38.25 g, respectively. The EA fraction of *H. cordata* (HCE) was redissolved in distilled water and used for evaluating hepatoprotective activities. Acute toxicity studies on HCE extract was performed at various doses (50, 100, 200 and 400 mg/kg) and was found that upto 400 mg/kg of HCE did not show any signs of toxicity in mice (data not shown). All reagents used in this study were of highest grade available commercially.

Carbon tetrachloride-induced hepatic injury:
The animals were randomly divided into four groups of six mice each. Group A served as the normal vehicle control and was administrated orally with 2 ml/kg corn oil daily for a period of 7 weeks. Group B orally administrated with 2 ml/kg body weight of CCl_4 (20% CCl_4 in corn oil) once a day for 7 weeks. The animals of Group C were pretreated with silymarin (200 mg/kg per day, dissolved into 0.1% DMSO) served as positive control and group D were pretreated with HCE extract (200 mg/kg per day) orally for 7 weeks, respectively before CCl_4 treatment. At the end of experiment, animals were sacrificed and the blood samples were collected into heparinized tubes for each group separately. Liver tissue was dissected out, washed immediately with ice cold saline, weighted and then immediately stored at -70° until analysis.

Biochemical assessment:
Liver damage was assessed by the estimation of serum activities of ALT, ALP and AST using commercially available test kits from BioVision (Milpitas, CA, USA) according to manufacturer's instructions. Total cholesterol, HDL-cholesterol, LDL/VLDL-cholesterol, TG levels were also estimated by using the respective diagnostic kits from BioVision.

Haematoxylin and eosin (H&E) staining:
A portion of the left lobe of the liver was preserved in 10% neutral formalin solution for at least 24 h, processed and paraffin embedded as per the standard protocol. Sections of 5 mm in thickness were cut, deparaffinised, dehydrated, and stained with H&E for the estimation of hepatocyte architecture.

Alkyl radical scavenging assay:
Alkyl radicals were generated by 2,2-azobis (2-amidinopropane) hydrochloride (AAPH, Sigma Chemical Co., St. Louis, MO, USA). Reaction mixtures containing 40 mM AAPH, 40 mM 4-POBN, and the indicated concentrations of tested samples diluted in PBS (pH 7.4) were incubated at 37° in a water bath for 30 min[17] and then transferred to 50 µl Teflon capillary tubes. The final concentration of HCE was 100 µg/ml. The spin adduct was recorded using the ESR spectrometer. The following measurement conditions were used: Central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW, gain 6.3×10^5, and temperature 298 K. The radical-scavenging activity

of the HCE at various concentrations was calculated according to the following formula: Scavenging rate=$(h_0-h_x)/h_0\times100\%$, where h0 and hx are the ESR signal intensities of samples with and without extract, respectively.

Hydroxyl radical scavenging assay:

Hydroxyl radicals were generated by the Fenton reaction and reacted rapidly with nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, Sigma Chemical Co., St. Louis, MO, USA). The resulting DMPO-OH adduct was detected by ESR[18]. The ESR spectrum was recorded 2.5 min after mixing with phosphate buffer solution (pH 7.4) supplemented with 20 µl of 0.3 M DMPO, 20 µl of 10 mM $FeSO_4$, and 20 µl of 10 mM H_2O_2 under the following conditions: Central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 1 mW, gain 6.3×10^5, and temperature 298 K. The radical-scavenging activity of the HCE at various concentrations was calculated according to the formula mentioned as in alkyl radical scavenging assay.

Statistical analysis:

All data are represented as the mean±SEM of at least three independent experiments. Statistical analyses were performed using SAS statistical software (SAS Institute, Cray, NC, USA) using Student's t-test and one-way analysis of variance, followed by Dunnett's multiple range tests. In all experiments *P* values less than 0.05 was considered statistically significant.

RESULTS

Effect of HCE extract on CCl_4-induced hepatotoxicity:

The serum biochemical data for the evaluation of CCl_4-induced hepatotoxicity by measuring the ALT and AST as conventional indicator of the liver injury are shown in fig. 1a-c. There was a remarkable elevation of serum

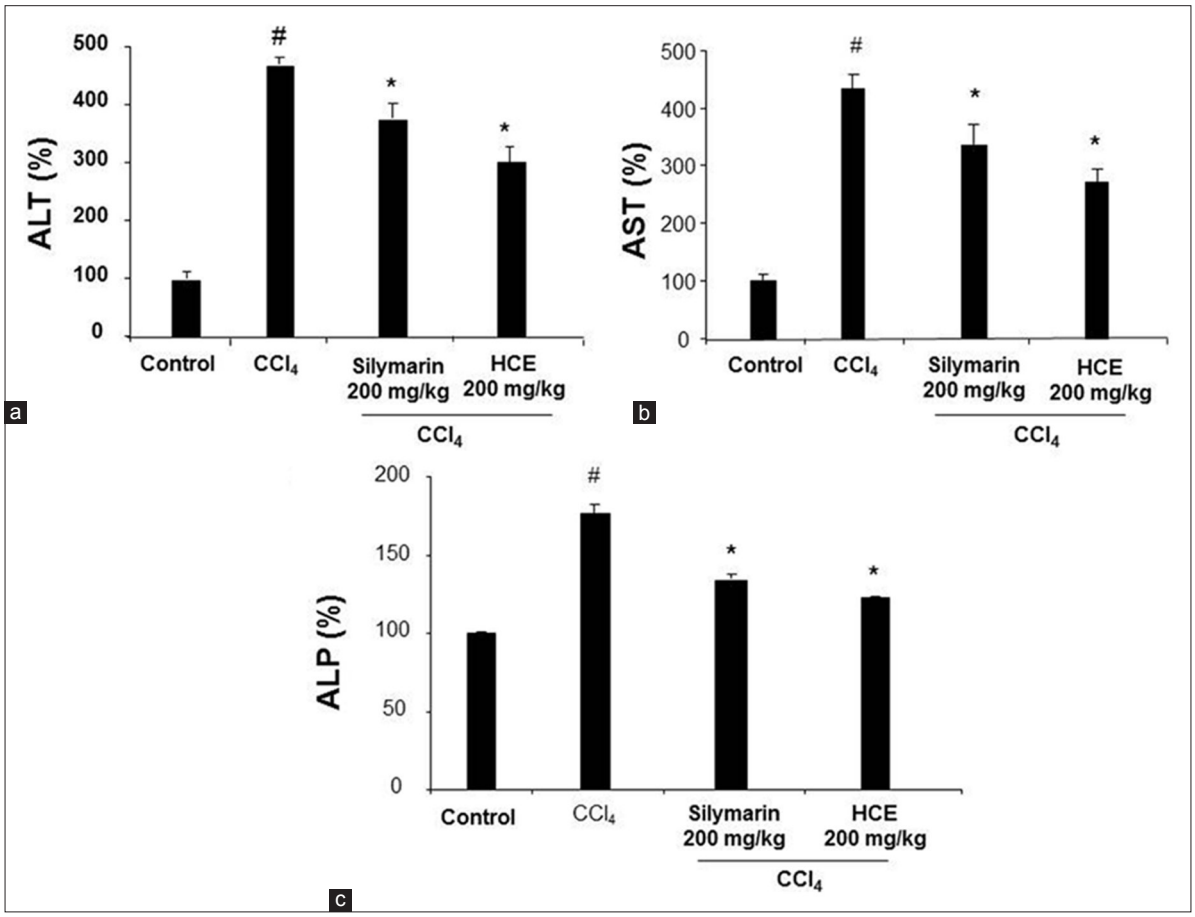

Fig. 1: Effects of HCE extract on serum ALT, AST, and ALP in CCl_4-treated mice.

Mice were administrated CCl_4 dissolved in corn oil (2 ml/kg, 10% v/v) orally for 7 weeks for chronic hepatic injury. Mice received silymarin (200 mg/kg) and HCE extract (200 mg/kg each) before CCl_4 treatment and the levels of ALT (a), AST (b) and ALP (c) are measured using commercial kits, respectively. Data are expressed as mean±SEM of six observations. $^{\#}P<0.05$, compared with control treated group and $^{*}P<0.05$, compared with CCl_4-treated group using one-way analysis of variance, followed by Dunnett's multiple range tests. HCE: *H. cordata* ethyl acetate extract, ALT: alanine aminotransferase, AST: aspartate aminotransferase, and ALP: alkaline phosphatase.

ALT, AST and ALP activities in the CCl_4-treated group as compared to the vehicle control (nearly, 5 fold for ALT (P<0.05), 4.5 fold for AST (P<0.05) and 1.8 fold for ALP (P<0.05), respectively indicating CCl_4-induced damage to the hepatic cells. However, administration of HCE extract at a dosage of 200 mg/kg suppressed the elevated serum ALT, AST and ALP in a significant manner when compared to CCl_4 alone treated group (P<0.05). The positive control silymarin treated group (200 mg/kg) also exhibited significant attenuation (P<0.05) when compared to CCl_4 treated groups. These results demonstrate that the levels of ALT, AST and ALP were significantly restored by treatment with HCE extract and the attenuation is on par with standard drug silymarin treated groups.

Effects of HCE extract on CCl_4-treated elevated lipid levels:

The effect of HCE extract on the cholesterol profile (total cholesterol, LDL/VLDL, HDL and TG) in CCl_4-induced liver injury in mice was shown in fig. 2a and b. A significant increase in the total cholesterol, LDL/VLDL and TG levels were observed in CCl_4-treated mice, compared to the control group (P<0.05). In contrast the HDL levels were decreased significantly in CCl_4-treated group. However, pre-treatment with HCE extract (200 mg/kg) significantly attenuated the CCl_4-induced increased serum levels of total cholesterol, LDL/VLDL and TG levels (P<0.05) indicating that HCE extract showed efficacy to possess antihyperlipidemic activity. Further, the CCl_4-induced decrease in HDL serum levels were reversed significantly to near normal conditions when pretreated with HCE extract (P<0.05). Silymarin (200 mg/kg) also attenuated the increased levels of total cholesterol, LDL/VLDL and TG and the decreased level of HDL, which was consistent with the results from other researchers (P<0.05)[19].

Effects of HCE on CCl_4-induced hepatic morphological changes:

The protective effect exerted by HCE extract against CCl_4-induced hepatotoxicity was further confirmed by conventional histological assessment (fig. 3). As shown in fig. 3a, the histology of the liver sections of control group showed normal hepatic cells. The stained sections of CCl_4 model group revealed extensive liver injuries characterized by moderate to severe hepatocellular hydropic degeneration and necrosis (fig. 3b). However, CCl_4-treated mice pretreated with 200 mg/kg silymarin or HCE extract (200 mg/kg) markedly ameliorated the hepatic lesions (fig. 3c and d), respectively, when compared with CCl_4 alone treated groups.

Alkyl free radical scavenging effects of HCE extract:

The alkyl free radical scavenging activity of the HCE extract was shown in fig. 4. The alkyl radical spin adduct of 4-POBN/free radicals was generated from AAPH at 37° after 30 min, and a decrease in the ESR signals was observed at increasing HCE

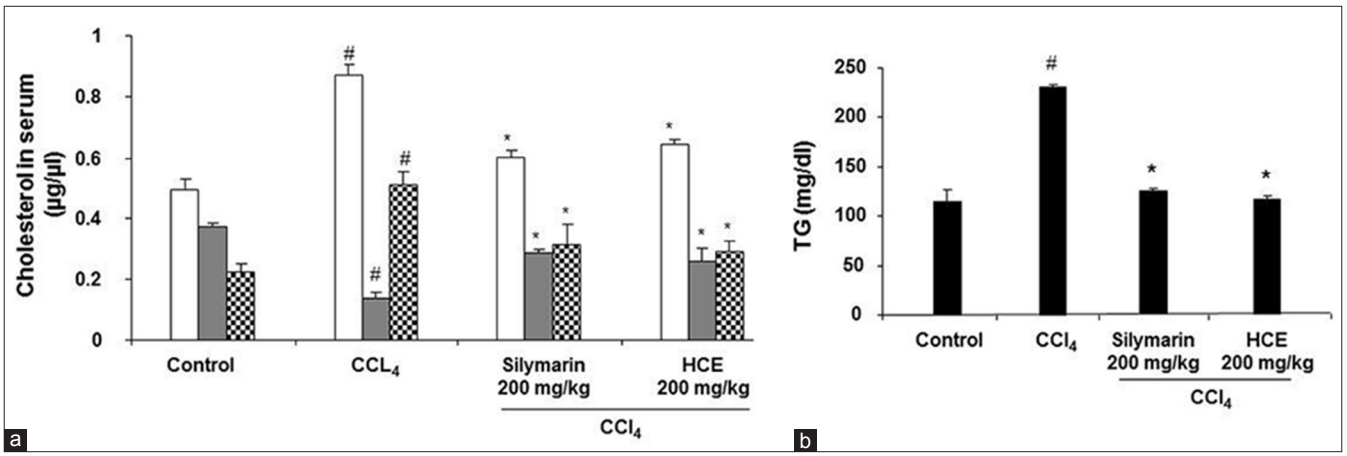

Fig. 2: Effects of HCE extract on serum lipid profile.

Mice were administrated CCl_4 dissolved in corn oil orally for 7 weeks for chronic hepatic injury. (a) Serum levels of total cholesterol, HDL and LDL/VLDL in acute liver injury. (b) Serum levels of TG in acute and chronic liver injury. Mice received silymarin (200 mg/kg) and HCE extract (200 mg/kg each) before CCl_4 intoxication and the serum levels were measured using commercial kits, respectively. Data are expressed as mean±SEM of six observations. #P<0.05, compared with control treated group and *P<0.05, compared with CCl_4-treated group using one-way analysis of variance, followed by Dunnett's multiple range tests. White bar: total cholesterol, grey bar: HDL, black checks bar: LDL/VLDL. HCE: *H. cordata* ethyl acetate extract, HDL: high density lipoprotein, LDL/VLDL: low density/very low density lipoprotein and TG: triglyceride.

concentrations. The alkyl radical scavenging activity was 90.5% at 100 µg/ml (fig. 4a), and the IC_{50} value of the HCE extract was 15.50 µg/ml. The decrease in the ESR signals for alkyl radical scavenging effect of HCE was shown in fig. 4b.

Hydroxyl radical scavenging effects of HCE extract:

The hydroxyl radical generated in the Fe^{2+}/H_2O_2 system was trapped by DMPO, which formed the spin adduct detected by ESR spectroscopy, and the typical 1:2:2:1 ESR signal of the DMPO−OH adduct was observed. The height of the third peak in the spectrum represents the relative amounts of DMPO−OH adduct. The addition of the HCE extract resulted in a decrease in the peak corresponding to the DMPO−OH adducts. The maximum hydroxyl radical scavenging effect of HCE was found to be 87.6% at 2 mg/ml and the IC_{50} value of HCE was determined to be 410 µg/ml (fig. 5a). The decrease in the ESR signals for alkyl radical scavenging effect of HCE was shown in fig. 5b. These results clearly demonstrate that the HCE possesses antioxidative activity and can effectively scavenge alkyl and hydroxyl radicals.

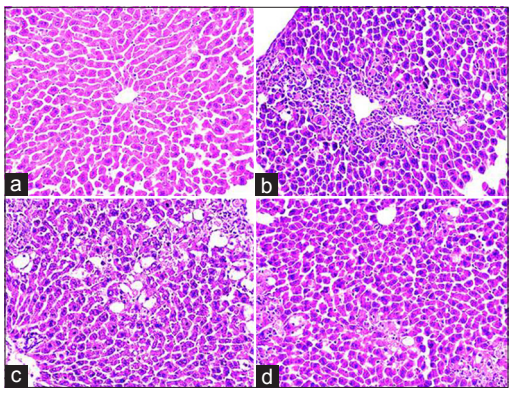

Fig. 3: Effects of HCE on hepatic morphological analysis.
Representative images at a magnification of 200 X of hematoxylin and eosin-stained liver sections. (a) Control group with no noticeable histological changes, (b) CCl_4-treated group showing severe histopathological changes like hydropic degeneration and necrosis, (c) Silymarin (200 mg/kg) and CCl_4 group, and (d) HCE (200 mg/kg) and CCl_4 group. HCE: *H. cordata* ethyl acetate extract, CCl_4: carbon tetrachloride.

DISCUSSION

This study was undertaken to demonstrate the protective ability of HCE extract against liver damage induced by CCl_4 in mice. Mounting evidence suggests that the elementary lesions caused by CCl_4-treatment in experimental animals are similar to those seen in most cases of human liver diseases[6,7]. Therefore this model has been used for decades to investigate and screen various natural and synthetic agents in the prevention and/or treatment of liver injuries. It was well documented that CCl_4 administration produce toxic free radicals thereby causing liver damage which further leads to the elevated levels of liver enzymes and altered levels of serum lipid profile disrupting the liver function[20]. Antioxidant rich natural herbs and their active constituents were well reported to exert beneficial effects in various disease conditions including liver diseases[14,15].

The ALT, AST and ALP, commonly referred as liver enzymes which are released from damaged hepatocytes and their levels in the serum have been widely recognized as a useful quantitative marker to study

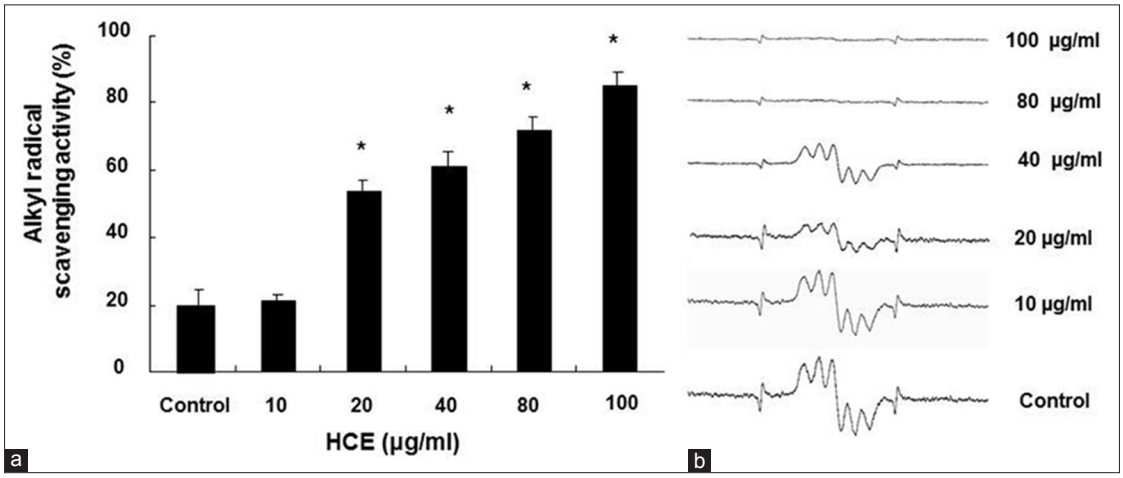

Fig. 4: Effect of HCE extract on alkyl radical scavenging activity.
(a) The capacities to scavenge the alkyl free radicals by different concentrations of HCE extract. (b) ESR spectra. Data are expressed as mean±SEM of six observations. *P<0.05 compared with untreated control group by Student's t-test. HCE: *H. cordata* ethyl acetate extract.

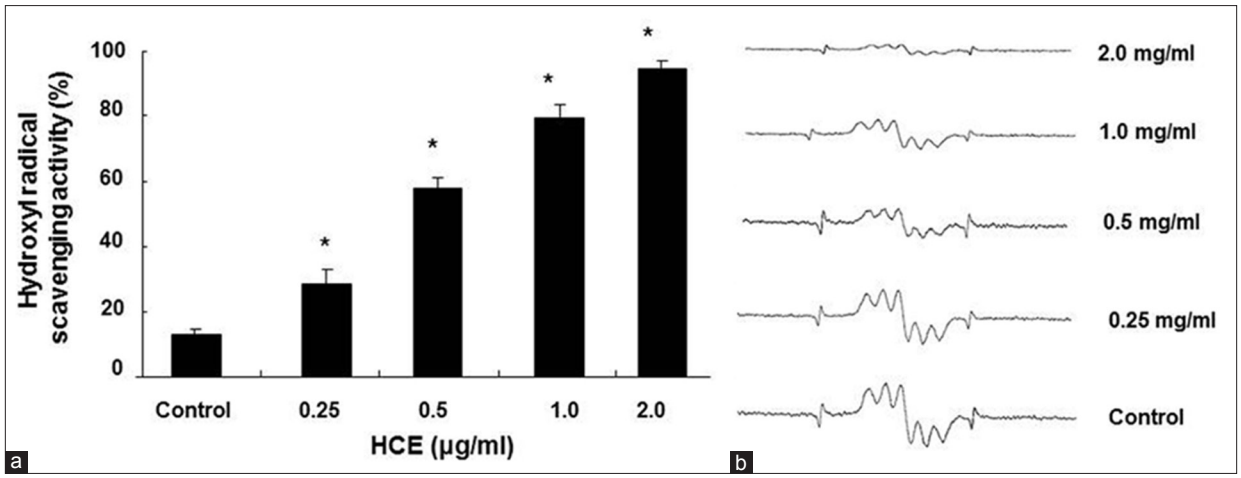

Fig. 5: Effect of HCE extract on hydroxyl radical scavenging activity.
(a) The capacities to scavenge the hydroxyl free radicals by different concentrations of HCE extract. (b) ESR spectra. Data are expressed as mean±SEM of six observations. *P<0.05 compared with untreated control group by Student's t-test. HCE: *H. cordata* ethyl acetate extract.

the extent and type of hepatocellular injuries[21]. In our experiment, HCE extract significantly attenuated the elevated serum levels of ALT, AST and ALP indicating that the proportion of damaged hepatocytes was reduced as a direct result of HCE administration.

Liver plays an important role in the regulation of lipoprotein transport in serum and cholesterol metabolism. During lipoprotein transport, LDL and HDL appear to be especially pivotal. LDL, rich in cholesterol and cholesterol ester, is regarded as bad cholesterol, whereas HDL contains relatively little cholesterol and is regarded as good cholesterol. Reports suggested that high levels of serum cholesterol, LDL, and TG and low levels of HDL were induced with CCl_4 in animal model systems[17]. Therefore, to evaluate whether HCE extract has any influence in hyperlipidemia induced by CCl_4, cholesterol catabolism was investigated. In the present study HCE extract significantly prevented CCl_4-induced liver damage as evidenced by reduced serum concentrations of cholesterol contents. Data form our study indicated that HCE extract restored the lipid levels to near normal levels.

The histopathological analysis provided complementary evidence that pretreatment with HCE extract attenuated the morphological changes in mouse liver tissue induced by CCl_4 administration. Examination of liver sections of mice, which received CCl_4, revealed disruption of the normal structural organization of hepatocytes compared to normal liver cells. Many hepatic cells were damaged and lost their characteristic appearance. In contrast treatment with HCE extract restored the hepatocellular architecture in CCl_4-induced mice.

Silymarin, a natural antioxidant was well reported to restore liver enzyme activities due to its antioxidant properties, chelating metal ions, inhibiting lipid peroxidation, protecting the membrane permeability properties, and preventing liver glutathione depletion in CCl_4-induced liver injury in mice[19]. In our present study, HCE extract showed similar or significantly greater effect when compared with the effects of silymarin at the same dose tested in CCl_4-induced liver damage in mice, indicating the potential of HCE extract in liver protection.

Earlier studies suggest that *H. cordata* inhibited lipidperoxidation in rat liver homogenates and possess antioxidant effects[22]. Recent report from our lab also indicated that *H. cordata* possess antineuroinflammatory activity by virtue of its antioxidant effects[23]. Data from our present work showed that HCE significantly inhibited alkyl and hydroxyl free radicals. Phytochemical studies on *H. cordata* showed that it contains several polyphenolic agents such as quercitrin, quercetin, rutin, hyperin, isoquercitrin, quercitrin, β-myrcene, β-pinene, α-pinene, α-terpineol and *n*-decanoic acid[24]. The antioxidant polyphenolic compounds present in HCE extract might be responsible for delivering such potent hepatoprotective actions in CCl_4-treated mice. In conclusion, our findings showed that HCE extract significantly prevented CCl_4-induced liver damage as evidenced by decreased serum activities of AST, ALT and ALP.

HCE extract also modulated serum concentrations of cholesterol contents. Further HCE exhibited potent antioxidant effects substantiating its beneficial effects in hepatoprotection.

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Accepted 27 April 2014
Revised 22 April 2014
Received 23 October 2013
Indian J Pharm Sci 2014;76(4):267-273