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Effects of White Radish (*Raphanus sativus*) Enzyme Extract on Hepatotoxicity

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Raphanus sativus (Cruciferaceae), commonly known as radish is widely available throughout the world. From antiquity it has been used in folk medicine as a natural drug against many toxicants. The present study was designed to evaluate the hepatoprotective activity of radish (*Raphanus sativus*) enzyme extract (REE) *in vitro* and *in vivo* test. The IC_{50} values of REE in human liver derived HepG2 cells was over 5,000 μg/m*l* in tested maximum concentration. The effect of REE to protect tacrine-induced cytotoxicity in HepG2 cells was evaluated by MTT assay. REE showed their hepatoprotective activities on tacrine-induced cytotoxicity and the EC_{50} value was 1,250 μg/m*l*. Silymarin, an antihepatotoxic agent used as a positive control exhibited 59.7% hepatoprotective activitiy at 100 μg/m*l*. Moreover, we tested the effect of REE on carbon tetrachloride (CCl_4)-induced liver toxicity in rats. REE at dose of 50 and 100 mg/kg and silymarin at dose of 50 mg/kg were orally administered to CCl_4-treated rats. The results showed that REE and silymarin significantly reduced the elevated levels of serum enzyme markers induced by CCl_4. The biochemical data were supported by evaluation with liver histopathology. These findings suggest that REE, can significantly diminish hepatic damage by toxic agent such as tacrine or CCl_4.

Key words: *Raphanus sativus*, Radish, Enzyme extract, Tacrine, CCl_4, Hepatoprotection

INTRODUCTION

Liver, an important organ actively involved in metabolic functions, is a frequent target of number of toxicants (Meyer and Kulkarni, 2001; Lee *et al*., 2004). Liver intoxication has increased as a result of exposure to high levels of environmental toxicants, so the liver has an important role in detoxification. Cytotoxins such as carbon tetrachroride (CCl_4), tacrine, acetaminophen, thioacetamide, rubratoxin B (Naga*s*hima *et al*., 2001) and H_2O_2 (Yang *et al*., 1999), along with lipopolysaccharide/D-galactosamine (Morita *et al*., 2003) have been investigated. In particular, tacrine is an acetylcholinesterase inhibitor that is approved for the treatment of Alzheimer's disease. However, tacrine treatment for Alzheimer's disease results in reversible hepatotoxicity in 30~50% of patients, which seriously limits its clinical use

(Watkins *et al*., 1994). Also CCl_4 has been widely used in animal models to investigate chemical toxin-induced liver damage (Recknagel and Lombardi, 1961). The most remarkable pathological characteristics of CCl_4-induced hepatotoxicity are fatty liver, cirrhosis and necrosis, which have been thought to result from the formation of reactive intermediates such as trichloromethyl free radicals ($CCl_3^•$ and/or $CCl_3OO^•$) metabolized by the mixed function cytochrome P450 in the endoplasmic reticulum (Recknagel, 1983). The CCl_3 radical alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage (Bishayee *et al*., 1995). Accordingly, the identification of constituents in natural products that have protective effects on tacrine or CCl_4-induced hepatotoxicity would be valuable.

A number of plants contain substances that can protect or treat hepatic injury and several agents with the ability to protect liver toxicity have been isolated from plants. One of these plants is radish which is gaining much attention. Radish (*Raphanus sativus*) is widely grown all over the world

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and has been used for more than 1,500 years. The radish is a root vegetable of the Brassicaceae family, commonly seen as a small-rooted, short-season vegetable, normally consumed in salads (Curtis, 2003). Different parts of radish including roots, seeds and leaves are used for medicinal purposes (Nadkarni *et al.*, 1976). Radish has been used ethnically as a laxative, stimulant, digestive aid, appetizer and in the treatment of stomach disorders (Kapoor, 2000). The main constituents of radish are 4-(methylthio)-3-butenyl isothiocyanate, allyl isothiocayanate, benzyl isothiocyanate and phenethyl isothiocyanate. It also contains flavonoids such as kaempherol glycosides, peroxidases and antioxidants (Wang *et al.*, 2004; Suh *et al.*, 2006; Hashimoto *et al.*, 2006). The previous studies reported that *R. sativus* extract showed antimicrobial (Esaki and Onozaki, 1982), antimutagenic (Hashem and Saleh, 1999) and anticarcinogenic effects (Hecht *et al.*, 2000). Lugasi *et al.* (1998) proved that radish root extract has an antioxidant activity *in vitro*. Takaya *et al.* (2003) suggested that crude extract of radish is also known to contain antioxidant enzyme activities and the antioxidant L-tryptophan was isolated from radish extract (Katsuzaki *et al.*, 2004). The radish root has an inhibitory effect on the membrane changes caused by a fat-rich diet and beneficially influences the natural scavenging activity of rat colon mucosa and protecting the cell membranes against lipid peroxidation (Sipos *et al.*, 2002). Recently, it was found that *R. sativus* extract did not show any toxic effects and could be considered as a potent hepatoprotectant (Salah-Abbès *et al.*, 2009) and ethanol extracts from *R. sativus* leaf powder was effective in decreasing transaminase, lactate dehydrogenase, alkaline phosphatase and total bilirubin (Anwar and Ahmad, 2006).

The aim of the present was to evaluate the hepatoprotective role of REE against cytotoxins inducing liver toxicity such as CCl_4 or tacrine.

MATERIALS AND METHODS

Chemicals and reagents. DMEM media and fetal bovine serum (FBS) were purchased from Gibco Ltd. (Grand Island, NY, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), tacrine and CCl_4 were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other solvents/chemicals were of reagent or analytical grade.

Preparation of REE. Freshly harvested fruit (or root) of white radish (*Raphanus sativus*) were crushed and the powdered radish were prepared for enzyme extraction. The enzyme extract of radish was treated with protease (Protamex™, Novo Nordisk Co., Bagsvaerd, Denmark) in a dried powder of radish. The powdered radish (100 g d.w.) were ultrasonicated with distilled water (1 : 2 ratio, w/v) for 30 min and it was added with 1% of protease (to dried
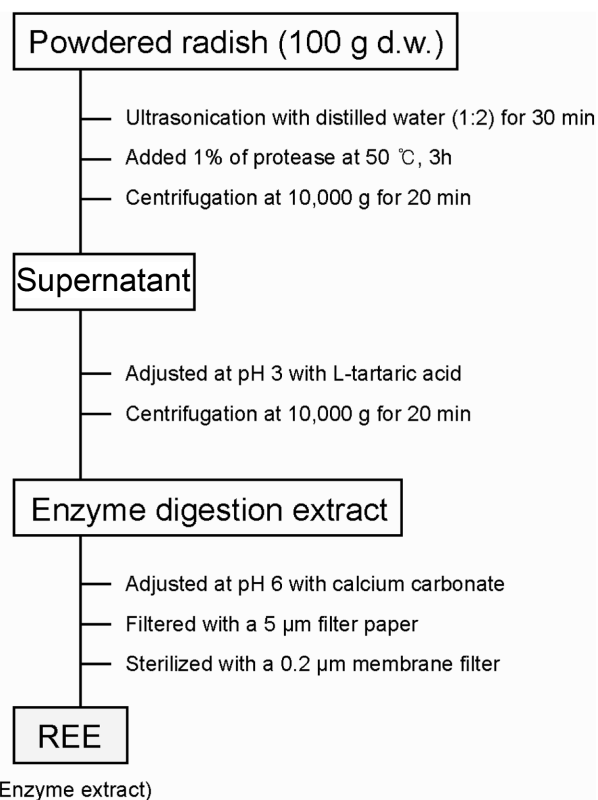

Fig. 1. Procedures for the preparation of REE. Extractive procedures were described in materials and method and showed extraction procedure by enzyme digestion.

weight of sample). The enzyme reactant was incubated at 50°C for 3 h and centrifuged at 10,000 *g* for 20 min. The supernatant was adjusted at pH 3 with L-tartaric acid until the supernatant becomes a transparent solution. After centrifugation at 10,000 *g* for 20 min, the supernatant was readjusted to pH 6 with calcium carbonate. The sample was filtered with Whatman filter paper (Particle retention; 5 μm) and then was filtered with 0.2 μm membrane filter (ADVANTEC MFS, Inc., CA, USA) (Fig. 1). The stock solutions were diluted appropriately with buffer or media at the time of testing.

Cell culture and treatment. HepG2 human hepatoma cell line was obtained from the ATCC (American Type Culture Collection) and the cells were cultured in complete DMEM (containing 10% fetal bovine serum (FBS), 100 units/m*l* of pencillin and 100 mg/m*l* of streptomycin at 37°C in 5% CO_2. After 10 passages, HepG2 cells were no longer used for the assays. The effect of REE on cytotoxicity was tested by treating cells with different concentrations of REE in DMEM medium.

Cell viability assay. The number of viable cells was determined by the ability of mitochondria to convert MTT

(3(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to formazan dye. HepG2 cells were cultured overnight in 96-well plates, at a density of 2×10^4 cells/200 μl in each well. The next day, the cells were coincubated with various concentrations of REE for 24 h. After incubation, the medium was removed and the cells were added with 10 μl of 10 mg/ml MTT into each well. After incubation for another 4 h at 37°C in a humidified 5% CO_2 atmosphere, MTT was removed, and cells lysed with 150 μl DMSO. The absorbance was measured at 550 nm using a microplate reader (OpsysMR, DYNEX. Ltd., Frankfurt, Germany).

In vitro assay of the hepatoprotective activity on tacrine-induced cytotoxicity in Hep G2 cells. Tacrine-mediated toxicity was evaluated by the MTT assay as previously described in cell viablilty assay. Briefly, HepG2 cells were maintained at 3×10^4 cell/well in complete medium consisting of DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml of pencillin and 100 mg/ml of streptomycin. Then incubated at 37°C in 5% CO_2 for 12 h. Cytotoxicity was assessed after a 2 h incubation period in the corresponding medium containing 1 mM of tacrine or without treatment (control). Seven different concentrations (78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 μg/ml) for REE were co-treated with 1 mM tacrine in triplicates. Silymarin (100 μg/ml) was used as a positive control.

Animals. Male SD rats, five weeks of age, were obtained from a Central Lab. Animal Inc. (Seoul, Korea) and housed at the animal maintenance facility of Clinical Research Center of Dong-A University Hospital. All animals were maintained in Specific Pathogen Free conditions according to OECD guidelines. All animal procedures were performed according to approved protocols (Approval number; DIACUC-11-33) from the Institutional Animal Care and Use Committee (IACUC) of Dong-A University and in accordance with recommendations for the proper use and care of laboratory animals.

Experimental design. Animals were divided into five groups consisting of six rats. Animals of groups I and II received olive oil (vehicle) for eight consecutive days. The rats of groups III and IV orally received REE for 8 days at a doses of 50 and 100 mg/kg/b.w., respectively, while animals in group V orally received silymarin at a dose of 50 mg/kg/b.w. for 8 days. All animals except group I were treated with CCl_4 (100 μl/100 g mixed 1 : 1 in corn oil) on the seventh day. Animals were killed under ether anesthesia at 48 h after of CCl_4 administration.

Serum biochemical assays. Blood was collected from blood vessels and kept for 30 min at 4°C. Serum was separated by centrifugation at 18,000 g for 15 min at 4°C. Serum biochemical parameters such as glutamate oxaloacetate

transaminase (GOT), glutamate pyruvate transaminase (GPT), triglyceride (TG) and total cholesterol (TC) were assayed in serum using commercial kit (Randox laboratories Ltd, Crumin, UK) in hematochemical analyzer (RAL Tecnica para el laboratoria, Barcelona, Spain).

Histopathological studies. Liver tissues were fixed in 10% buffered formaldehyde and processed for histological examination by conventional methods and stained with hematoxylin and eosin (H&E). The liver pathology was scored as described by French *et al.* (2000) as follows; 0 = no visible cell damage; 1 = focal hepatocyte damage on < 33% of tissue; 2 = focal hepatocyte damage on < 33-66%
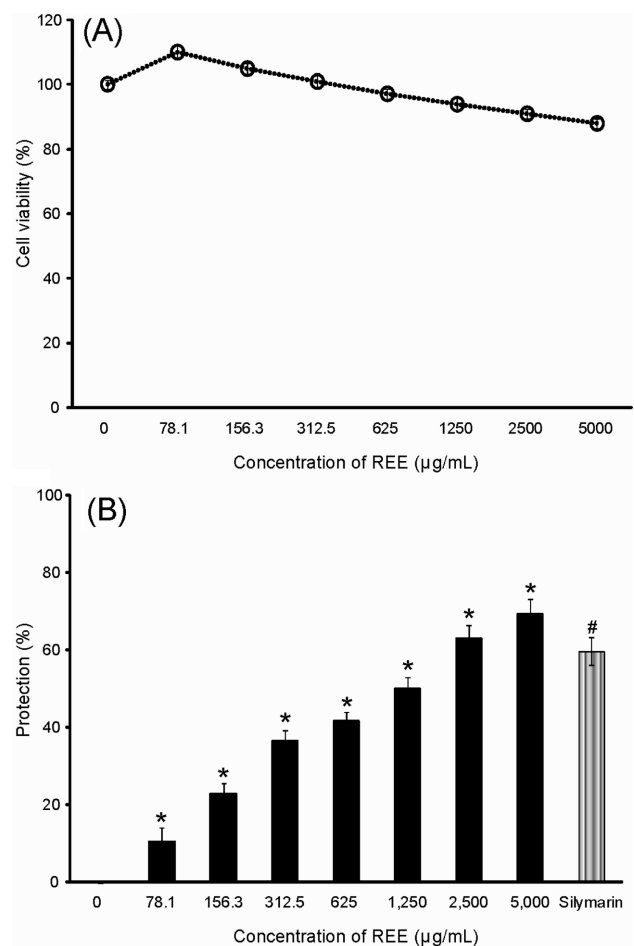

Fig. 2. Hepatoprotective effect of REE on tacrine-induced cytotoxicity in Hep G2 Cells. (A) For the Hep G2 cells viability, cells were treated for 24 h with various concentrations of REE and the cell viability was evaluated by MTT assay. (B) Tacrine-induced cytotoxicity was assessed after incubation for 2 h with different concentrations of REE in the presence 1 mM of tacrine. Silymarin (100 μg/ml) was used positive control. The results are expressed as percentages of control, and the data are the mean ± SD of at least three determinations. * and #, $p < 0.05$ as compared to the untreated group.

of the tissue; 3 = extensive, but focal, hepatocyte lesion; 4 = diffuse hepatocyte necrosis. The histopathological examinations of treatment groups were carried out blindly.

Statistical analysis.   All data are expressed as mean and standard deviation (S.D.). The evaluation of statistical significance was performed using Student t-test or one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, Version 18.0 (Chicago, IL, USA). The criterion for statistical significance is expressed as $p < 0.05$.

RESULTS

Effect of REE on cell viability.   To exclude the possibility that inhibitory effect of REE on hepatoprotective activity might be caused by the inhibition of HepG2 cell growth, we compared the number of cell growth in the presence and absence of REE. We observed the cell viability with MTT assay after treatment of REE with different concentrations. After treatment, 50% of cell viability presenting concentration (IC$_{50}$ values) of REE was over 5,000 μg/ml in tested maximum concentration (Fig. 2A). Therefore, in this study, we tested hepatoprotective activity on tacrine-induced cytotoxicity in Hep G2 cells at concentrations below IC$_{50}$ of REE.

Effect of REE on hepatoprotective activity in HepG2 cells.   In the present study, we investigated the hepatoprotective activity of REE on tacrine-induced cytotoxicity in HepG2 cells by MTT assay. REE showed hepatoprotective activity in dose-dependent manner and the value of the 50% effective concentration (EC$_{50}$) of REE were 1,250 μg/ml. In addition REE induced inhibition of 69.2% in cell toxicity at 5,000 μg/ml (Fig. 2B). Silymarin, well known for its hepatoprotective efficiency, was used as a reference sub-
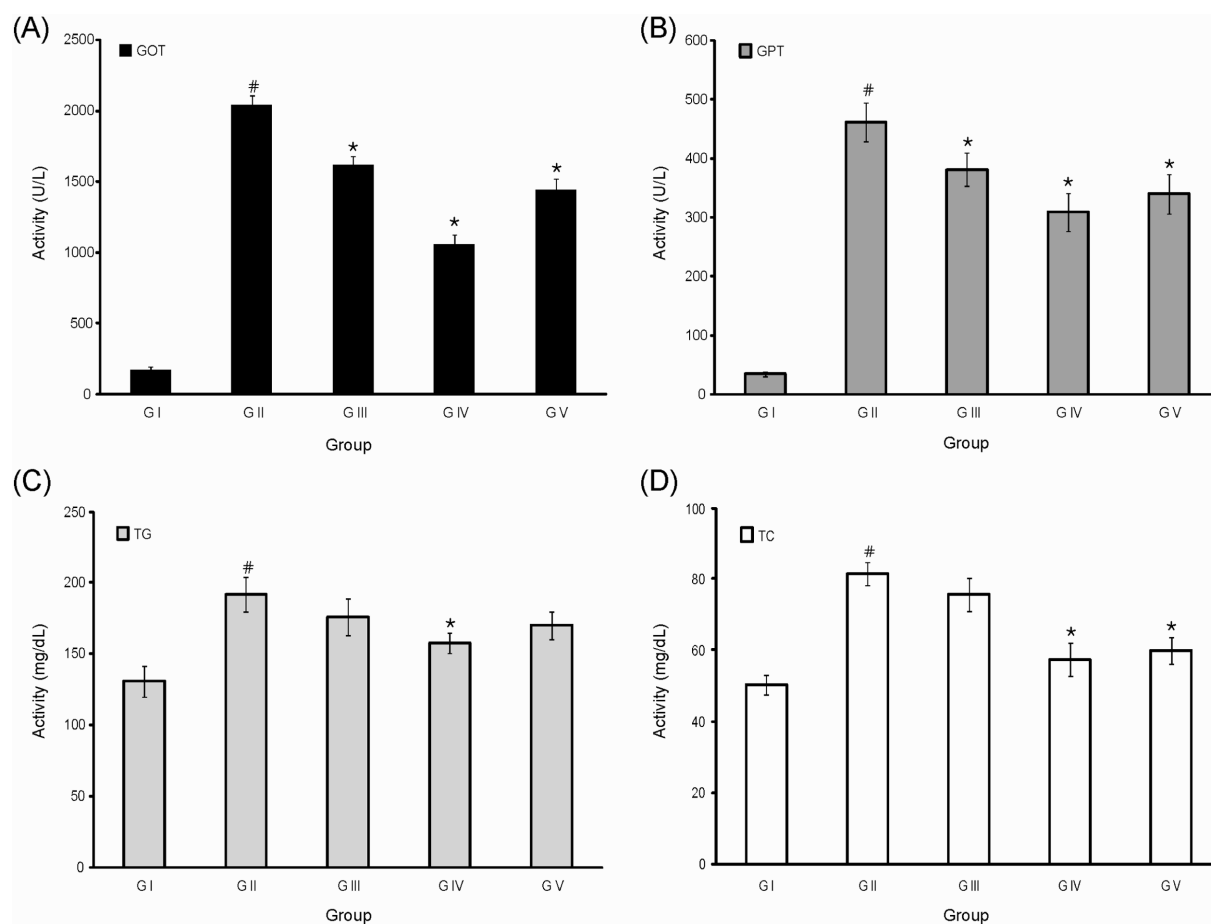

Fig. 3. Effect of REE on activities of serum (A) GOT, (B) GPT, (C) TG and (D) TC in CCl$_4$-treated SD rats. Animals of GI and II group received olive oil (vehicle) for eight consecutive days. The rats of GIII and IV group orally received REE orally at a dose of 50 and 100 mg/kg, respectively while animals in GV group orally received silymarin at a dose of 50 mg/kg orally for 8 days. All animals except GI group were treated with CCl$_4$ (100 μl/100 g mixed 1 : 1 in corn oil) on the seventh day. Animals were killed under ether anesthesia after 48 h. The data represent the mean ± SD of six rats. $^{\#}$, $p < 0.05$ as compared to GI group (vehicle) and *, $p < 0.05$ as compared to GII group (CCl$_4$ alone).

stance, and showed protective effect of 59.7% at 100 μg/ml (Fig. 2B). These results showed that the effective concentration of REE showed little cytotoxicity to HepG2 cells.

Effect of REE on serum biochemical enzyme activities. The effect of the REE pretreatment on the CCl$_4$-induced elevation of the activities of serum GOT, GPT, TG and TC is shown in Fig. 3. Administration of CCl$_4$ significantly elevated the release of GOT, GPT, TG and TC activities in serum ($p < 0.05$). Pretreatment of rats (group III and IV) with REE significantly prevented the elevation of GOT (Fig. 3A) and GPT (Fig. 3B) at both doses used. On the other hand, REE reduced TG (Fig. 3C) and TC (Fig. 3D) in a dose dependent manner. Moreover, silymarin (group V) significantly diminished the enhanced levels of all marker enzymes (GOT, GPT, TG and TC) as compared with CCl$_4$ alone group. Our data demonstrated that REE at high dose (100 mg/kg/b.w.) resulted in a significant improvement in all biochemical parameters towards the normal values.
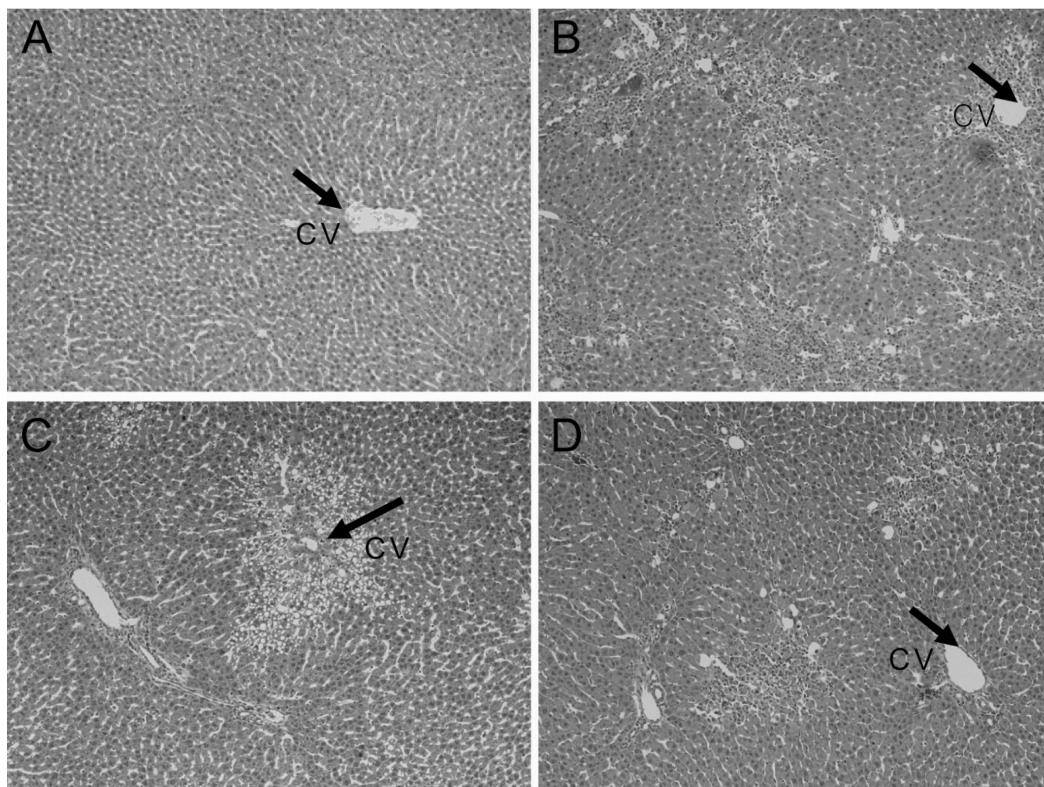

Fig. 4. Effect of REE on CCl$_4$–induced liver damage of SD rats. Photomicrographs represented histopathological changes in liver tissue. (A) GI group showed normal liver architecture and (B) GII group were orally administered orally by olive oil to CCl$_4$-treated rats. (C) GIV and (D) GV group were administered orally by REE (100 mg/kg/b.w) or silymarin (50 mg/kg /b.w) to CCl$_4$-treated rats, respectively. Specimens were stained with hematoxylin and eosin. Magnification 100×.

Table 1. Histological injury score of liver under different dose of REE

Group			Dose (mg/kg)	NO.	Injury of Score	
					Necrosis	Inflammation
Vehicle		Group I	-	6	0	0
CCl$_4$ treatment	Vehicle	Group II	-	6	5 ± 0.5	4 ± 0.7
	REE	Group III	50	6	4 ± 1.2	3.5 ± 1.5
		Group IV	100	6	2.5 ± 0.8	3 ± 1.2
	Silymarin	Group V	50	6	2.5 ± 1.2	3 ± 0.6

Livers were scored for hepatic injury via light microscopy with score 0 = no visible cell damage; score 1~3: focal hepatocyte damage on less than 33% of the tissue; score 4~6: focal hepatocyte damage on more than 33~66% of the tissue; score 7~9: global hepatocyte necrosis. The data represent the mean ± SD of 6 rats.

Effect of REE on histopathological changes. Histopathological studies provided supportive evidence for the biochemical analysis. The liver sections of normal control animals (group I) showed hepatic cells with well preserved cytoplasm, prominent nucleus and central vein (Fig. 4A). The photomicrographs of the liver in rats of CCl_4 alone group (group II, Fig. 4B) showed severe necrosis or inflammation. However, the REE-treated group (group IV, 100 mg/kg/b.w.) (Fig. 4C) and silymarin-treated group (group V, 50 mg/kg/b.w.) (Fig. 4D) reduced necrosis or inflammation in comparison with CCl_4 alone group (group II). In addition this was confirmed by histopathological evaluation (Table 1). On the other hand, there was no significant difference in the relative liver weight among groups (data not shown). These results demonstrated that REE had a protective effect on CCl_4-induced hepatic damage.

DISCUSSION

Tacrine toxicity has been examined in microsomes (Madden *et al.*, 1993), monolayers of primary rat and human hepatocytes (Galisteo *et al.*, 2000), HepG2 cells (Viau *et al.*, 1993) and the V79 Chinese hamster cell line (Berson *et al.*, 1996). In the present study, the hepatoprotective activity of REE on tacrine-induced cytotoxicity in HepG2 cells was evaluated. The value of the 50% effective concentration (EC_{50}) of REE were $1,250\ \mu g/ml$. Silymarin, well documented for its hepatoprotective efficiency, was used as a reference substance, and showed 59.7% at $100\ \mu g/ml$. Our results indicated that REE effectively reduced the tacrine-induced hepatotoxicity in HepG2 cells and REE also was safe because it induced little cytotoxicty at the effective dose.

Moreover CCl_4-induced hepatotoxicity in rats represents an adequate experimental model of fibrosis/cirrhosis in man and it is used for the screening of hepatoprotective drugs (Al-Shabanah *et al.*, 2000). The liver represents the principal site of toxicity, although it induces sublethal proximal tubular injury in the kidney and focal alterations in granular pneumocytes (Striker *et al.*, 1968). In general, the estimation of the serum enzyme is always a useful quantitative marker of the extent and class of liver damage (Sreelatha *et al.*, 2009). Significant elevation in the activities of serum hepatospecific enzymes was seen when hepatocellular damage leads to abnormalities of liver function (Gutiérrez and Solís, 2009). It has been reported that these enzymes (GOT, GPT, TG and TC) exhibit higher activity in CCl_4-induced fatty liver (Gutiérrez and Solís, 2009; Wolf, 1999).

Damage to the cell integrity of the liver by CCl_4 is reflected by an increase in the activity of GOT, which is released into circulation after cellular damage. GPT is an ectoenzyme of the hepatocyte plasma membrane. Acute and chronic toxicity increased permeability of the hepatocyte membrane and cellular leakage (Paduraru *et al.*, 1996). In the present study, we demonstrated the role of REE in protecting against CCl_4-induced hepatotoxicity *in vivo* model and we used silymarin as positive control. Silymarin has been reported to prevent liver injuries induced by various chemicals or toxins including ethanol or CCl_4 (Farghali *et al.*, 2000; Lieber *et al.*, 2003). It is reported to have a protective effect on the plasma membrane of hepatocytes (Ramellini and Meldolesi, 1976). As a potent scavenger, it prevents lipid peroxidation and normalizes the lipid profile of hepatocyte membranes (Muriel and Mourelle, 1990). In addition silymarin has been reported to provide liver protection against CCl_4-induced liver damage in rat models (Mourelle *et al.*, 1989) and the levels of serum enzymes namely GOT and GPT were reduced after treatments of rats with silymarin (Yadav *et al.*, 2008). The present study revealed that administration of CCl_4 alone significantly raises the serum level of enzymes like (GOT and GPT) in rats (Gutiérrez and Solís, 2009) as observed in our results. However, the increased serum levels of GOT and GPT induced by CCl_4 were decreased by the administration of REE and silymarin. This suggested that REE can protect against membrane fragility and decrease of leakage of the marker enzymes into the circulation.

Levels of TG and TC in the liver also have been estimated to explain the status of liver. High level of TG and TC in the liver is the indication of the liver injury. Previous reports indicated that TC and TG increased in CCl_4-induced fatty liver (Seakins and Robinson, 1963; Torres-Durán *et al.*, 1998). According to Recknagel and Lombardi (1961), the accumulation of TG in liver of CCl_4-treated rats is not due to the interference with the TG formation by the liver, but the inhibition or destruction of TG secreting mechanism. TC and TG level has been increased in the CCl_4-induced group (group II), and has been decreased by REE (group III and IV) or silymarin (group V) treatment. Thus, the observed restoration of the CCl_4-evoked changes in the lipid profile of serum showed the protective nature of REE.

These findings were further confirmed by a comparative histoarchitectural examination of the liver sections from different groups of treated rats. Liver histopathology after CCl_4 administration revealed inflammation, fatty degeneration and severe necrosis of hepatocytes. It is provoked by the increased production of a highly reactive intermediate of CCl_4 like trichloromethyl free radical, which is normally detoxified by endogenous glutathione and enzymatic antioxidants but in excess it may deplete glutathione stores and the status of other antioxidants, allowing the reactive intermediate to react with and destroy the hepatic cells (Pessayre *et al.*, 1985). Thus, CCl_4-induced hepatic fibrosis/cirrhosis rat model has been useful in studying the effects of hepatoprotective drugs with therapeutic potential to be used in humans (Heller *et al.*, 2000). Various pharmacological and chemical substances which belong to the intrinsic or idiosyncratic group of hepatotoxins may induce a level of hepatic damage varying from asymptomatic hepatic func-

tional disturbance to widespread hepatic fibrosis/cirrhosis (Klaassen *et al.*, 1990). Silymarin has been histopathologically shown to have significant anti-inflammatory effect on hepatic tissue and is effective in reversing hepatic fibrosis due to CCl_4-induced damage (Guyton *et al.*, 2006; Jeong *et al.*, 2005). In the current study, hepatic fibrosis/cirrhosis was successfully induced by CCl_4 injection in rats and these results were obtained similar to previous study (Guyton *et al.*, 2006). In the silymarin and REE with CCl_4 injection group, the presence of connective tissue was almost normal around the central veins and accumulation of inflammatory cells around central veins was lower than in the CCl_4 treated group. Indeed, histopathological results reconfirmed the changes in serum enzyme activities which reflected the extent of cell damage. These results suggest that REE can prevent hepatic damage through the suppression of inflammation in CCl_4-induced rat hepatic fibrosis/cirrhosis. However, it is unclear whether REE play a central role in hepatic fibrosis/cirrhosis, which may be involved in inflammatory process.

In conclusion, we have shown that REE has a protective effect on tacrine-induced hepatotoxicity in HepG2 cells and the administration of REE prevented biochemical and histomorphological alteration induced by CCl_4. The findings support the use of REE for the improvement of liver disorders because enzyme extraction is safer than methanol extraction in toxicity.

ACKNOWLEDGEMENTS

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