# Antioxidant Effects of Aqueous Extract of *Terminalia chebula* *in Vivo* and *in Vitro*

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**The ripe fruit of *Terminalia chebula* RETZIUS (*T. chebula* RETZ) (Combretsceae), which is a native plant in India and Southeast Asia, has traditionally been used as a popular folk medicine for homeostatic, antitussive, laxative, diuretic, and cardiotonic treatments. The objective of this study was to evaluate the protective effects of an aqueous extract of fruit of *T. chebula* on the *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative injury observed in cultured rat primary hepatocytes and rat liver. Both treatment and pretreatment of the hepatocytes with the *T. chebula* extract (TCE) significantly reversed the *t*-BHP-induced cell cytotoxicity and lactate dehydrogenase leakage. In addition, TCE exhibited *in vitro* ferric-reducing antioxidant activity and 2,2-diphenyl-1-picryhydrazyl free radical-scavenging activities. The *in vivo* study showed that pretreatment with TCE (500 or 1000 mg/kg) by gavage for 5 d before a single dose of *t*-BHP (0.1 mmol/kg i.p.) significantly lowered the serum levels of the hepatic enzyme markers aspartate aminotransferase and alanine aminotransferase and reduced the indicators of oxidative stress in the liver, such as the glutathine disulfide content and lipid peroxidation, in a dose-dependent manner. Histopathologic examination of the rat livers showed that TCE reduced the incidence of liver lesions, including hepatocyte swelling and neutrophilic infiltration, and repaired necrosis induced by *t*-BHP. Based on the results described above, we speculate that TCE has the potential to play a role in the hepatic prevention of oxidative damage in living systems.**

**Key words** hepatotoxicity; antioxidant; oxidative stress; *Terminalia chebula* extract; *tert*-butyl hydroperoxide

It has been proposed that oxidative stress damage to cellular and extracellular macromolecules, such as proteins, lipids, and nucleic acids[1—3] results from the tipping of balance toward prooxidant status. The production of reactive oxygen species (ROS) has been implicated in the pathogenesis of age-related diseases[4,5] such as cancer and coronary heart disease and neurodegenerative disorders such as Alzheimer's disease.[6] It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea, and herbs may contribute to shift the balance toward an adequate antioxidant status.[7] The dried ripe fruit of *Terminalia chebula* RETZIUS (Combretaceae), which is a native plant in India and Southeast Asia, is commonly known as black myroblans in English and harad in Hindi and has traditionally been used as a popular folk medicine for homeostatic, antitussive, laxative, diuretic, and cardiotonic treatments.[8,9] *T. chebula* exhibits *in vitro* antioxidant and free radical-scavenging activities.[10] Its antimicrobial,[11] antiviral,[12,13] anticancer,[14] antianaphylaxis,[15] and antidiabetic[16] activities have been reported. However, to the best of our knowledge, the biological effects of *T. chebula* have so far not been extensively investigated, and it is not known whether *T. chebula* can inhibit or decrease liver damage induced by oxidative stress. In the present study, we examined the protective potential of *T. chebula* extract (TCE) against *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative hepatocyte injury in rat primary hepatocyte cultures and rat liver. Various biochemical measurements, such as antioxidant parameters including free radical-scavenging activity, cytotoxicity, lipid peroxidation, and glutathione (GSH) status were assessed as an index of oxidative stress.

## MATERIALS AND METHODS

**Materials** Leibovitz's L-15 (L-15) medium was obtained from Gibco Life Technologies. Percoll was purchased from Amersham Biosciences. Type I collagenase was obtained from Worthington. Bovine serum albumin (BSA), 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT), streptomycin, penicillin, insulin, dexamethasone, galactose, sodium selenite, *t*-BHP, 2,2-diphenyl-1-picrylhydrazyl (DPPH), pyruvate, $\beta$-NADH, and kits for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from Sigma. All other chemicals were of analytical grade.

**Analytical Procedures** The medicinal dried ripe fruit of *T. chebula*, which was purchased locally (Kyungdong Herb Market, Seoul, Korea), was identified by Dr. B. W. Kang (College of Life and Environmental Sciences, Korea University). The dried fruits were ground with a mortar and then soaked in distilled water (300 g/4 l) followed by refluxing for 3 h and cooling. The undissolved materials were removed by passing though a Whatman 41 filter paper (Clifton, NJ, U.S.A.). The filtrate was freeze-dried, yielding a final amount of exsiccated TCE of approximately 70 g. The total polyphenol (TP) content was measured using Folin-Ciocalteu reagent.[17] To each tube, 0.1 ml of TCE was added followed by 2 ml of methanol and 0.2 ml of 2 N Folin-Ciocalteu reagent. After 3 min, 1 ml of 15% $Na_2CO_3$ was added. The tubes were mixed thoroughly, kept for 2 h at room tempera-

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ture, and read at 765 nm using gallic acid as a standard, with the results expressed as gallic acid equivalents (GAE). The dissolved TCE in dimethyl sulfoxide (DMSO) (0.1 mg/ml) was added to an equal volume of a solution of 0.2% $AlCl_3 \cdot 6H_2O$ in ethanol. The mixture was vigorously shaken, and absorbance was read at 367 nm after 10 min at room temperature. Flavonoid contents were expressed in milligrams of catechin equivalents (CE) per gram of extract.[17]

The ferric-reducing antioxidant power (FRAP) assay measures the antioxidant potentials of "antioxidants" to reduce the $Fe^{3+}$/2,4,6-tripyridyl-*s*-triazine (TPTZ) complex present in a stoichiometric excess to the blue colored $Fe^{2+}$ form. The FRAP reagent containing 2.5 ml of 10 mM TPTZ solution in 40 mM HCl, plus 2.5 ml of $FeCl_3 \cdot 6H_2O$ and 25 ml of 0.3 M sodium acetate buffer at pH 3.6 was freshly prepared by mixing together the different reagents. The FRAP assay was performed according to the standard method.[18] Briefly, 900 $\mu$l of FRAP reagent was mixed with 60 $\mu$l of methanol and 30 $\mu$l of either the test sample, the standard, or an appropriate reagent blank. The absorbance was read at 595 nm after 4-min incubation at 37 °C on a JASCO spectrometer (V-530, Tokyo, Japan) equipped with a water circulator to maintain the temperature. A calibration curve of ferrous sulfate (250—2000 $\mu$M) was used, and the results expressed in units of $\mu$mol $FeSO_4 \cdot 7H_2O\ g^{-1}$ dry matter (DM) with three determinations.

The radical-scavenging activities of the extracts were evaluated by assessing their DPPH-scavenging activity.[19] DPPH was dissolved in ethanol to give a 100 $\mu$mol/l solution. Then, 100 $\mu$l of a test compound (stock concentration 1 mg/ml) in distilled water was added to 900 $\mu$l of ethanolic DPPH solution. The mixtures were shaken vigorously and left in the dark for 30 min. The decrease in DPPH absorption was measured at 517 nm. $EC_{50}$ values calculated denote the concentration of sample required to scavenge 50% DPPH. The results are expressed as $\mu$g DM $ml^{-1}$. Ascorbic acid was used as a positive control.

The laboratory animals were treated in compliance with the *Guide for the Care and Use of Laboratory Animals* (Committee on Care and Use of Laboratory Animals, 1985). Male Sprague-Dawley rats (body weight 200±10 g) were purchased from Samtako Bio Korea Co. (Gyeonggi, Korea), and allowed free access to a standard diet (Samyang-Feed Co. Ltd., Inchon, Korea) and tap water.

Rat hepatocytes were prepared by collagenase perfusion, as described previously[20,21] with several modifications. The rats were anesthetized by the intraperitoneal administration of ketamine hydrochloride (5 mg/kg) and xylazine hydrochloride (2 mg/kg), and initially the liver was perfused at 37 °C *via* the portal vein with 200 ml of perfusion medium, hank's balanced salt solution (pH 7.4) containing penicillin/streptomycin (100 U) at a rate of 20 ml/min. After 7 to 8 min, the liver was perfused with 200 ml of the same medium supplemented with 0.05% type I collagenase, 4 mM $CaCl_2$, and trypsin inhibitor (5 mg) for another 10 min at a rate of 13 ml/min. To collect a single-cell suspension of hepatic parenchymal cells, the liver was removed, washed with phosphate-buffered saline (PBS), sieved through a nylon mesh into a flask, and washed with L-15 medium at pH 7.5, supplemented with 18 mM HEPES, 0.2% BSA, 0.05% glucose, and insulin (5 $\mu$g/ml). The cell suspension was centrifuged at 200 rpm for 3 min and washed again. The cell pellet was resuspended in Percoll solution and centrifuged at 1500 rpm for 15 min to remove the dead cells and nonparenchymal cells. Then, the pellet containing the live cells was resuspended and washed twice. The isolated hepatocytes were plated on collagen type 1-precoated plates and cultured in L-15 medium (pH 7.6) supplemented with 18 mM HEPES, 0.2% BSA, insulin/transferring (5 $\mu$g/ml), 1 $\mu$M dexamethasone, galactose (5 mg/ml), sodium selenite (5 ng/ml), penicillin (100 IU/ml), and streptomycin sulfate (100 $\mu$g/ml). Cell viability was determined using tryptophan blue dye exclusion and was found to be greater than 90%.

The experiments described in this study were performed without any noticeable differences observed in the results when using cells obtained from the various livers. The cells were incubated in a humidified incubator at 37 °C (Vision, Korea) in an air atmosphere. The medium was replaced with fresh identical medium 4 h after plating. At 20 h after plating, the media were replaced with L-15 media containing the test chemicals. The cells were either seeded onto 24-well plates at $1.8 \times 10^5$ cells/well for cytotoxicity studies [MTT assay and lactate dehydrogenase (LDH) leakage], or 6-well plates at $9 \times 10^5$ cells/well for malondialdehyde (MDA) measurement. All treatments were performed 20 h after cell attachment to allow for monolayer formation. After cell attachment, the hepatocytes were washed and then incubated in L-15 medium for 30 min with TCE (0.1, 1, 10, 100, and 1000 mg/ml) and 1.5 mM *t*-BHP. For the control group, neither TCE nor *t*-BHP was added. For the experiment on pretreatment with TCE, 16 h after cell attachment, cells were treated with TCE (0.1, 1, 10, 100, and 1000 mg/ml) for 4 h, washed, and then incubated in L-15 medium with 1.5 mM *t*-BHP for 30 min. The medium was removed and assayed for the leakage of LDH by measuring the absorbance change at 340 nm in an assay medium containing 136 mM pyruvate, 19 mM NADH, and Kebs-Henseleit buffer containing 2% albumin. The cell viability was measured with the MTT assay.[22] A volume of 240 $\mu$l of MTT (1 mg/ml) in L-15 medium was added to each well, followed by incubation for 3 h at 37 °C. The formazan crystals formed in the actively metabolizing cells were extracted with 10% SDS (in 1 N HCl) and the absorbance at 540 nm was recorded.

The intracellular reduced GSH and glutathine disulfide (GSSG) of the hepatocytes and liver tissues were determined using HPLC, as described by Reed *et al.*[23] MDA, the lipid peroxidation product in the cells, was assayed using a thiobarbituric acid fluorometric method, with the excitation measured at 515 nm and emission at 552, using 1,1,3,3-tetramethoxypropane as the standard.[24] The protein concentration was determined by the method of Lowry *et al.*,[25] using BSA as the standard. The rats were divided into three groups (six rats/group). To investigate the hepatoprotective activity against *t*-BHP-induced oxidative stress, TCE (500, and 1000 mg/kg) was administered to the animals daily by gavage for 5 consecutive days. On day 5, 0.1 mmol/kg of *t*-BHP was injected (i.p.) into each animal, 18 h later the rats were killed by cervical dislocation, and blood samples collected from the vena cava caudalis for the assays of AST and ALT. Immediately after collecting the blood, the livers were excised and rinsed in PBS. A small section of each liver was placed in 10% phosphate-buffered formalin to be used in the histo-

Table 1. Total Polyphenol Content (TP), Expressed as Gallic Acid Equivalents (GAE), Flavonoid Content, Expressed as Catechin Equivalents (CE), 2,2-Diphenyl-1-picrylhydrazyl Free Radical (DPPH˙)-Scavenging Activity, and Ferric-Reducing Antioxidant Power (FRAP) in TCE[a)]

| TP (g GAE $kg^{-1}$ DM) | Flavonoid content (mg CE $kg^{-1}$ DM) | DPPH˙ $EC_{50}$ ($\mu$g DM $ml^{-1}$)[b)] | FRAP (mmol $FeSO_4 \cdot 7H_2O$ $g^{-1}$ DM) |
|---|---|---|---|
| 169.5±10.6 | 436±12 | 127.1±1.8 | 2.4±0.6 |

[a)] Each value is mean±S.D. of three replicate experiments. Different letters indicate significantly different values ($p<0.05$) (vertical comparison). DM, dry matter. [b)] Amount of sample necessary to decrease the initial DPPH concentration by 50%.

chemical analysis. A portion of the remaining liver was frozen in liquid nitrogen and stored at −80 °C for later biochemical analysis. The hepatic enzymes AST and ALT were used as the markers for early acute hepatic damage. The serum activities of AST and ALT were determined using the colorimetric method.[26)] Immediately after their removal from the animals, the hepatic tissues were fixed in 10% buffered formaldehyde. The formaldehyde-fixed tissue samples were embedded in parafilm, and 5-$\mu$m sections were cut and processed for histologic examination according to the conventional methods and stained with hematoxylin and eosin (H&E). The results obtained are expressed as mean±standard deviation (S.D.). Student's $t$-test was used to make a statistical comparison between the groups by one-way analysis of variance. Significant differences were set at the level of $p<0.05$.

## RESULTS

The $EC_{50}$ values for the radical-scavenging activity of TCE, its TP content and flavonoid content, expressed as GAE and CE, respectively, and the FRAP are shown in Table 1. The TCE contained comparable levels of phenolic compounds (169.5±10.6 g GAE $kg^{-1}$ DM) and flavonoid compounds (436±12 mg CE $kg^{-1}$ DM). The DPPH $EC_{50}$ value for the TCE (127.1±1.8 $\mu$g/ml), which was used to measure its radical-scavenging ability, and the FRAP value of TCE (2.4±0.6 mmol $FeSO_4 \cdot 7H_2O$ $g^{-1}$ DM), which was used to measure the antioxidant potential, were 3.6-fold and 11-fold lower those of ascorbic acid (35.5±1.4 $\mu$g/ml, 25.5±0.32 $\mu$mol $FeSO_4 \cdot 7H_2O g^{-1}$ DM, respectively).

The TCE was further evaluated using the rat primary hepatocyte system, and its cytotoxicity was assessed by incubating the cells with doses of the extract of up to a dose of 1000 $\mu$g/ml. At the maximum dose of TCE, no cytotoxicity was found, since cell viability remained above the level of the control after 30 min of incubation (Table 2).

Primary hepatocyte cultures were exposed to increasing concentrations of $t$-BHP from 0 to 1.8 mM for 30 min, and MTT was measured as an index of cell toxicity. The cytotoxic effect of $t$-BHP was found to be dose dependent (Table 3). The oxidative stress induced by 1.5 mM $t$-BHP caused more than 43% cell death after 30 min of incubation, together with a 3.6-fold increase in LDH leakage (Table 3). The treatment of the cells with different concentrations of TCE in the presence of 1.5 mM $t$-BHP protected the hepatocytes against the cytotoxicity of $t$-BHP in a dose-dependent manner (Fig. 1). At the dose of 1000 $\mu$g/ml, the $t$-BHP-treated cells showed fully recovered cell viability from 55% in cells treated with $t$-BHP. LDH leakage was also strongly suppressed. Inhibition (70% for MTT, $p<0.05$, and 250 U/ml for LDH, $p<0.01$) was also observed at TCE (10 $\mu$g/ml). To determine whether TCE exerts its protective effects intracellularly rather than extracellularly by reacting with $t$-BHP in the culture medium, we investigated the effects of TCE pretreatment on $t$-BHP-induced hepatocyte damage. Pretreating

Table 2. Cytotoxicity of TCE Assessed by the Microculture Tetrazolium (MTT) Assay in Primary Cultured Rat Hepatocytes

| TCE ($\mu$g/ml) | % Viability of control (MTT) |
|---|---|
| 1000 | 122.0±7.1 |
| 100 | 102.8±0.4 |
| 10 | 104.2±1.3 |
| 1 | 104.7±1.9 |
| 0.1 | 105.8±1.0 |

Cells were treated with TCE for 30 min in L-15 medium supplemented with HEPES under the conditions described in Materials and Methods. Cell viability was determined in the MTT assay. Results are expressed as mean±S.D. ($n=3$ or more).

Table 3. Cytotoxicity of *tert*-Butyl Hydroperoxide ($t$-BHP) Assessed by Microculture Tetrazolium (MTT) Assay and Lactate Dehydrogenase (LDH) Assay in Primary Cultured Rat Hepatocytes

| Treatment | % Viability of control (MTT) | U/ml (LDH) |
|---|---|---|
| Control | 100±5.6 | 64.6±14.6 |
| $t$-BHP (0.3 mM) | 84.4±2.4 | 144.3±14.3 |
| $t$-BHP (0.6 mM) | 74.0±4.1 | 168.2±28.8 |
| $t$-BHP (0.9 mM) | 63.1±3.8 | 245.0±33.1 |
| $t$-BHP (1.2 mM) | 59.9±2.8 | 307.1±14.9 |
| $t$-BHP (1.5 mM) | 56.7±0.7 | 359.4±39.4 |
| $t$-BHP (1.8 mM) | 46.0±1.2 | 442.6±20.6 |

Hepatocytes cultures were treated with various doses of $t$-BHP for 30 min. Data represent mean±S.D. ($n=3$).

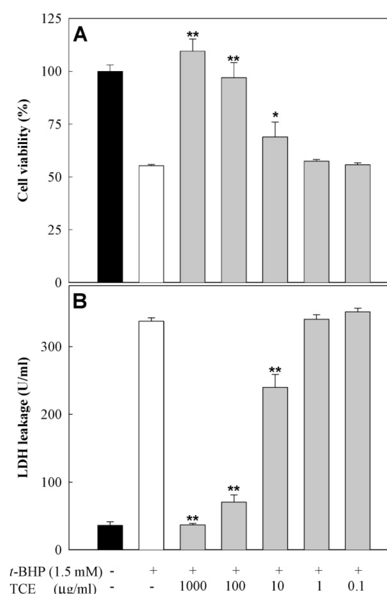


Fig. 1. Liver Postprotective Effects of TCE on $t$-BHP-Induced Oxidative Hepatotoxicity in Cultured Rat Hepatocytes

Values are expressed as mean±S.D. ($n=3$). *$p<0.05$, **$p<0.01$, compared with cells treated with $t$-BHP alone.

the cells with TCE for 4 h prior to 1.5 mM *t*-BHP treatment recovered cell viability from 56% in cells treated with *t*-BHP to 98%, and LDH leakage was also strongly suppressed (Fig. 2).

The hepatic enzymes AST and ALT were used as biomarkers for early acute hepatic damage. A single dose of *t*-BHP (0.1 mmol/kg) i.p. elevated rat serum AST and ALT levels and increased the formation of MDA in the liver (Table 4). Pretreatment with TCE significantly suppressed these acute hepatotoxicity reactions induced by *t*-BHP (Table 4). GSH is known to play a protective role against *t*-BHP-induced toxicity,[27] and the oxidative stress of tissues generally involves the GSH system. Therefore we measured the level of GSH for each group of livers. While the administration of *t*-BHP significantly reduced the level of GSH ($p<0.05$) and significantly increased that of GSSG ($p<0.05$), pretreatment with TCE significantly protected against this reduction in the level of GSH depletion and normalized the GSSG increase produced by *t*-BHP (Table 4).

Treatment with *t*-BHP caused neutrophil infiltration, swelling of liver cells, and cell death in rat livers (Fig. 3). However, based on microscopic examinations, the severe hepatic lesions induced by *t*-BHP were reduced by the administration of TCE (500, and 1000 mg/kg) (Figs. 3C, D), with the results well correlated with those of the serum AST and ALT measurements (Table 4). Cell death was markedly inhibited by pretreatment with TCE (1000 mg/kg).

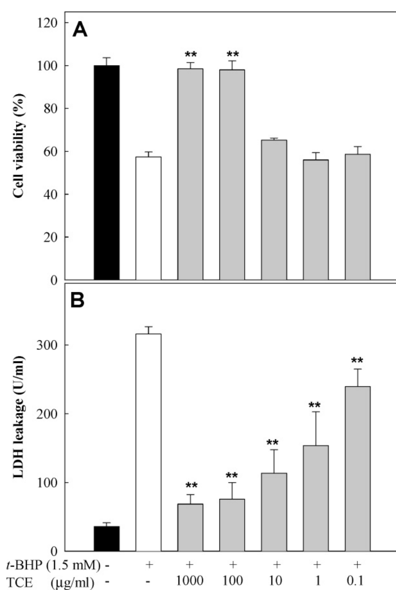


Fig. 2. Liver Preprotective Effect of TCE on *t*-BHP-Induced Oxidative Hepatotoxicity in Cultured Rat Hepatocytes

Values are expressed as mean±S.D. ($n=3$). ** $p<0.01$, compared with cells treated with *t*-BHP alone.

## DISCUSSION

Although the active principle(s) in TCE for its antioxidative activity was not investigated in this study, ellagic acid, 2,4-chebulyl-$\beta$-D-glucopyranose, chebulinic acid, casuarinin, chelanin, and 1,6-di-*O*-galloyl-$\beta$-D-glucose have been reported recently to be active constituents in *T. chebula* fruits.[10,14] The major roles of these active components have been investigated focusing on antioxidant activity. A few studies investigated the biologic activities of *T. chebula*. A methanol extract of *T. chebula* had cytotoxic effects in several malignant cell lines including human cell lines of breast cancer, osteosarcoma, and prostate cancer.[14] Oral administration of the methanolic extract of *T. chebula* (100 mg/kg body weight) reduced the blood sugar level in normal and in alloxan (120 mg/kg) diabetic rats. The water-soluble fraction of *T. chebula* (0.01—1.0 g/kg) showed significant inhibitory effects on systemic and local anaphylaxis in rats and mice. However, to our knowledge, the biologic activities of *T. chebula* against hepatic toxicity have not been studied. Liver cells are active in the metabolism of exogenous chemicals, and this is a major reason why the liver is a target.[28] When detoxifying xenobiotics, ROS are generated to cause oxidative stress.[29,30] We employed *t*-BHP, a short-chain analogue of lipid peroxide, to induce acute oxidative stress in rat hepatocytes and the *in vivo* rat liver. Two distinctive pathways are involved in the metabolism of *t*-BHP in hepatocytes. The first employs the microsomal cytochrome P-450 system leading to the production of ROS such as peroxyl and alkoxyl radicals that initiate lipid peroxidation,[31] while the second involves the conversion of GSH peroxidase to *t*-butanol and oxidized GSSG. GSSG is then reduced to GSH by GSH reductase, resulting in NADPH oxidation. Decreased GSH and oxidized NADPH contribute to altered $Ca^{2+}$ homeostasis, which is considered to be a major event in *t*-BHP-induced toxicity.[32] The *in vitro* experiment conducted here showed that TCE was able to quench DPPH free radicals. In the hepatotoxicity experiment, TCE exhibited antioxidative and protective activity against the injury induced by *t*-BHP, as reflected in increased cell viability (MTT assay and LDH release) (Table 3). The cytoprotective activities of other common antioxidants such as the prophenol epigallocatechin gallate (EGCG) and the flavonoid quercetin in MTT assay were 1.2- and 1.4-fold higher (data not shown), repectively, than

Table 4. Effects of TCE on Serum Enzymes Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) Activities and Hepatic Malondialdehyde (MDA) and Glutathione (GSH) Levels in Rats Treated with *t*-BHP

| Treatment | AST (IU/l) | ALT (IU/l) | MDA (mmol/g liver) | Glutathione (nmol/mg protein) | |
|---|---|---|---|---|---|
| | | | | Reduced | Oxidized (GSSG) |
| Untreated control | 114.5±8.1 | 12.0±7.3 | 63.5±6.2 | 70.5±3.7 | 23.1±3.9 |
| *t*-BHP (0.1 mmol/kg) | 381.1±24.3*b)* | 49.3±4.5*b)* | 133±20*b)* | 59.2±0.2*d)* | 69.7±2.6*d)* |
| TCE*a)* (500 mg/kg) | 148.9±25.6*c)* | 35.6±5.0*c)* | 79.4±8.8*c)* | 69.8±10 | 18.3±3.9*e)* |
| (1000 mg/kg) | 112.1±7.7*c)* | 14.7±4.4*c)* | 63.9±9.5*c)* | 65.7±16 | 17.3±3.4*e)* |

*a)* Animals were pretreated with various concentrations of TCE by gastric tube for 5 consecutive days before the administration of *t*-BHP. The rats were killed 18 h later. Then, the serum AST and ALT, and hepatic MDA and GSH were determined. Values are expressed as mean±S.D. ($n=6$). *b)* $p<0.01$, compared with untreated control. *c)* $p<0.01$, compared with the group treated with *t*-BHP alone ($n=6$). *d)* $p<0.05$, compared with untreated control. *e)* $p<0.05$, compared with the group treated with *t*-BHP alone ($n=6$).

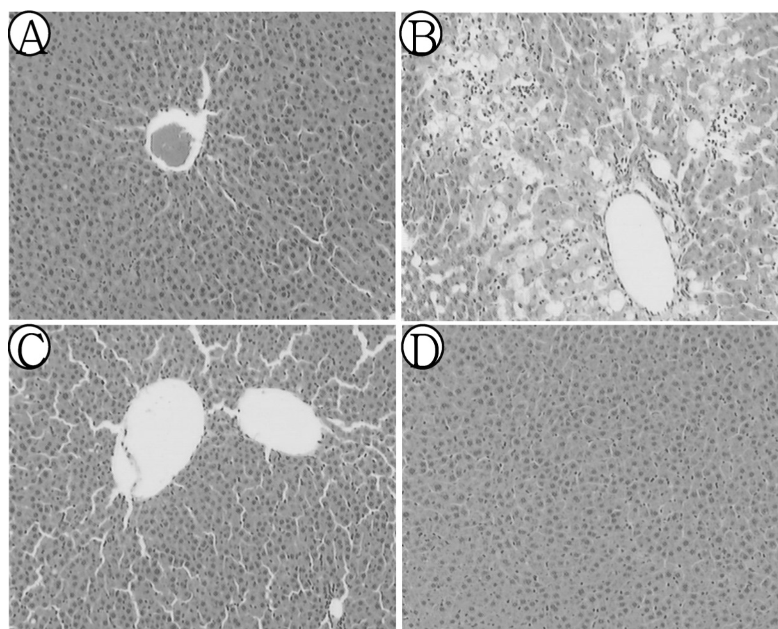


Fig. 3. Effects of TCE Pretreatment on *t*-BHP-Induced Liver Damage

The rats were pretreated with TCE (500 or 1000 mg/kg, i.g.) once daily for 5 consecutive days. The control rats were given saline. Three hours after the final treatment, rats were treated with *t*-BHP (0.1 mmol/kg, i.p.). (A) Solvent control group ($n=6$). (B) Animals treated with *t*-BHP showed spotty liver cell death with neutrophilic infiltrates (arrows) and severe ballooning degeneration of hepatocytes (asterix). (C) Animals pretreated with 500 mg/kg of TCE, and then with *t*-BHP. (D) Animals pretreated with 1000 mg/kg of TCE, and then *t*-BHP. Hematoxylin/eosin staining; original magnification ×200.

that of TCE. The *in vivo* study showed that *t*-BHP reduced the GSH level and increased the GSSG level in rat liver (Table 4), although TCE blocked these effectively (Table 4). In this study, the hepatoprotective effects of TCE against *t*-BHP might result from its ability to scavenge ROS and attenuate the loss of GSH. The significant effectiveness of pretreatment and subsequent removal of the TCE prior to *t*-BHP treatment (Fig. 2) indicated that TCE exerted its protective activity intracellularly, rather than extracellularly by reacting with *t*-BHP in the culture medium.

The findings of the present study suggest that *T. chebula* fruits have potent antioxidative and protective effects against *in vitro* free radical generation and *t*-BHP-induced oxidative hepatotoxicity in rat primary cultured hepatocytes and rat liver. Further studies designed to isolate, identify, and characterize their active antioxidant constituents(s) should provide a greater understanding of the mechanisms underlying the antioxidant effects of TCE.

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