ANTI-OXIDATIVE AND HEPATOPROTECTIVE ACTIVITIES OF *DENDROBIUM TOSAENSE* AND *EPHEMERANTHA FIMBRIATA* IN CARBON TETRACHLORIDE-INDUCED ACUTE LIVER INJURY

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This study was intended to investigate the anti-oxidative activity, anti-oxidative substance content and the protective effect of *Dendrobium tosaense* (DT) and *Ephemerantha fimbriata* (EF) on carbon tetrachloride-induced acute liver injury. Firstly, we detected the contents of total polyphenols and flavonoids in the DT_{EtOH} and EF_{EtOH}. Second, the anti-oxidative activities of the DT_{EtOH} and EF_{EtOH} were determined using DPPH free radical scavenging assay, reducing power assay, ferrous metal-chelating capacities assay *in vitro*. Finally, the hepatoprotective effect of DT_{EtOH} and EF_{EtOH} was determined using CCl_4-induced acute liver injury. The activities of serum alanine aminotransferase (sALT), serum aspartate aminotransferase (sAST), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), the levels of glutathione (GSH) and malondialdehyde (MDA) were measured. The results were confirmed by liver histology. DT_{EtOH} and EF_{EtOH} possessed anti-oxidative activity and polyphenol content. When mice were treated with CCl_4 in the absence of DT_{EtOH} and EF_{EtOH}, the activities of sALT and sAST, and MDA level were increased, while the activities of antioxidant enzymes (SOD, GPx, catalase, GR, GST) and GSH level were decreased. When the mice were treated with CCl_4 in the presence of DT_{EtOH} and EF_{EtOH}, the activities of sALT and sAST, and MDA level were significantly decreased, while the activities of antioxidant enzymes, and the GSH level were increased. The above results were confirmed by liver histological examination. DT_{EtOH} and EF_{EtOH} possessed anti-oxidative activity and protected against CCl_4-induced acute liver injury in mice by increasing the anti-oxidant enzymes activities and GSH level.

Key words: *Dendrobium tosaense, Ephemerantha fimbriata,* anti-oxidative activity, acute liver injury, MDA

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Introduction

Liver injury caused by hepatotoxins, such as carbon tetrachloride (CCl_4), ethanol, and acetaminophen, is characterized by varying degrees of hepatocyte degeneration and cell death[1]. The generation of reactive intermediate metabolites from the metabolism of hepatotoxins, and the occurrence of reactive oxygen species (ROS) during the inflammatory reaction account for a variety of pathophysiologic pathways leading to cell death, such as covalent binding, depletion of glutathione (GSH) and protein thiols and associated lipid peroxidation[2, 3]. In recent years, the clinical importance of the herbal drugs treatment for liver inflammation has received considerable attention.

CCl_4 is a xenobiotic that produces hepatotoxicity in human as well as in various experimental animals[4,5]. Covalent binding of the metabolites of CCl_4, trichloromethyl free radicals, to cell proteins is considered to be the initial step in a chain of events that eventually lead to membrane lipid peroxidation and finally to cell death[6]. The general strategy for prevention and treatment of liver damage includes reducing the production of reactive metabolites by using antioxidants[7]. Antioxidants appear to act against diseases by raising the levels of endogenous defense (e.g., by up-regulating gene expressions of the antioxidant enzymes, such as SOD, catalase, and GPx and GR) [8,9].

Several medicinal plants have been screened based on the integrative approaches on drug development from Ayurveda[10]. Herba Dendrobii, the stem of several *Dendrobium* species (Orchidaceae), locally known as ‘Shi-Hu’ is used in traditional Chinese medicine for antipyretic, eyes-benefiting, immunomodulatory, anti-inflammatory, and antioxidant activities[11-14]. Some polyphenols and flavonoid isolated from *Dendrobium* plants are the major components for anti-oxidative effect[13, 15]. Moreover, the *D. moniliforme*, a major source of Herba Dendrobii, is very expensive and a kind of endangered rare crude drugs. Therefore, it is worthy of searching for new medical resources of Herba Dendrobii that could be substituted for *D. moniliforme*.

The natural antioxidants in complex mixtures ingested with the diet are more efficacious than pure compounds in preventing oxidative stress-related pathology due to particular interactions and synergisms[2]. Based on the excellent antioxidant activities of *D. aphyllum*, it was worthy of evaluating antioxidant activities of the two species. In present study, we compare the anti-oxidative activity of the two species using DPPH free radical scavenging assay, reducing power assay, ferrous metal-chelating capacities assay *in vitro*. Second, we estimated the total flavonoid and total polyphenol contents of the two species using HPLC. Finally, male ICR mice were orally treated with DT, EF or silymairn (as standard reference) for three days and accompanied CCl_4 administration at the last administration. Hepatic GSH and MDA levels as well as activities of AST and ALT in serum and catalase, SOD, GR, and GPx in liver tissues were measured to monitor liver injury. The extent of CCl_4-induced liver injury was also analyzed through liver histopathological observations.

Materials and Methods

I. Preparation of the extracts

Dendrobium tosaense Makino (DT) was supply from the Chiayi Agricultural Experiment Station, Taiwan Agricultural Research Institute. *Ephemerantha* *fimbriata* (Bl.) Hunt *et* Summerh (EF) was purchased from Lien-Ho TCM drug store in Taichung. DT and EF were identified by Dr. Chao-Lin Kuo, leader of the School of Chinese Medicine Resources (SCMR). The voucher specimen was deposited at SCMR.

The dried powders (50 g) of the DT and EF were extracted by using 3 L of 70% ethanol for 24 h a cycle for four times. The extracts were filtered, combined and concentrated under reduced pressure at 40°C to obtain extract of DT_{EtOH} and EL_{EtOH}. The yield ratios of DT_{EtOH} (2.57 g) and EL_{EtOH} (15.20 g) extracts were 5.14% and 30.40%, respectively.

II. Chemicals and drugs

DPPH, potassium ferricyanide, trichloroacetic acid, ferric chloride glutathione, $FeCl_2$, ferrozine, aluminum nitrate, potassium acetate, quercetin, Folin-Ciocalteu (FC) reagent, sodium carbonate, H_2O_2, glutathione (GSH), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), 1,1,3,3-tetraethoxypropane (TEP), butylated hydroxytoluene (BHT), silymarin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Carbon tetrachloride, Sulfuric acid, trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were purchased from Merck Chemical Co. (Darmstadt, Germany). Biochemistry assay kits purchased from Randox Laboratories Ltd (UK). CCl_4 was dissolved in olive oil as 0.2% (v/v) solution. Silymarin was suspended in 0.5% CMC. All other chemicals and reagents used were obtained from local sources and were of analytical grade.

III. Animals

Male ICR mice (20 ± 2 g), obtained from the animal central of college of medicine in National Taiwan University, were housed in standard cages at a constant temperature of 22 ± 1°C, relative humidity 55 ± 5% with 12 h light–dark cycle (08:00 to 20:00) for 1 week at least before the experiment.

Animals used in this study were housed and cared in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Committee on Animal Research, China Medical University, under the code 2006-14-N.

IV. Experimental design

(I) DPPH free radical scavenging assay

The DPPH free radical scavenging ability was determined according to the method of Saha *et al.*[16]. Different concentrations of the extract (30.5–488.25 μg/ml) and the ascorbic acid standard (11.7–125 μg/ml) were placed in different test tubes. The extract or ascorbic acid (30 μL) was mixed with 120 μl of 100 mM Tris-HCl and then mixed with 150 μL of ethanol solution containing the DPPH radical (50 μM), shaken vigorously, and left to stand for 20 min in the dark. The reduction of the DPPH radicals was measured by an ELISA reader at 517 nm. Radical scavenging activity was measured as the decrease in DPPH absorbance and the inhibition percentage was calculated by using the following equation:

$$\text{Scavenging activity (\%)} = (1 - A_{517} \text{ of sample} / A_{517} \text{ of control}) \times 100\%$$

(II) Reducing power (RP) assay

RP was determined according to the method of Oyaizu[17] with slight modifications. Sample (50 μl), sodium phosphate buffer (50 μl, 0.2 M, pH 6.6) and potassium ferricyanide (50 μl, 10 mg/ml) were mixed and incubated at 50°C for 20 min. Trichloroacetic acid (50 μl, 10 mg/ml) was added to the mixture and centrifuged at 6,000 ×g for 10 min. The supernatant (80 μl) was mixed with distilled water (50 μl) and ferric chloride (50 μl, 1.0 mg/ml), and then its absorbance was measured at 700 nm. The capability of the sample to reduced action was calculated using the following equation:

$$RP (\%) = (A_{700} \text{ of sample} / A_{700} \text{ of } 0.1 \text{ mg/ml}^{-1} \text{ GSH}) \times 100\%$$

(Ⅲ) Ferrous metal-chelating capacities assay

The Fe_2^{+}-chelating ability was determined according to the method of Haro-Vicente *et al.*[18] with slight modifications. The Fe^{2+} was monitored by measuring the formation of ferrous iron-ferrozine complex at 562 nm. Sample was mixed with 2 mM $FeCl_2$ and 5 mM ferrozine at a ratio of 10:1:1. The mixture was shaken and left to stand at room temperature for 10 min. The absorbance of the resulting solution at 562 nm was measured. The lower is absorbance of the reaction mixture, the higher is Fe^{2+}-chelating ability. The capability of the sample to chelate the ferrous iron was calculated using the following equation:

$$\text{Chelating effect } (\%) = (1 - A_{562} \text{ of sample} / A_{562} \text{ of control}) \times 100\%$$

(Ⅳ) Determination of total flavonoid content

Flavonoid content was determined according to the method of Jia *et al.*[19] with slight modification. An aliquot of 1 ml of the solution containing 10 mg extracts in methanol was added to test tubes containing 0.1 ml of 10% aluminum nitrate, 0.1 ml of 1 M potassium acetate and 2.8 ml of water. After 40 min at room temperature, the absorbance was read spectrophotometrically at 415 nm. Quercetin was used as a standard. The concentrations of flavonoid compounds were calculated from a quercetin standard curve.

(Ⅴ) Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent according to the method of Kujala *et al.*[20] with a slight modification. Briefly, the two extracts (0.5 ml) was mixed with 0.5 ml of FC reagent (previously diluted 50% with distilled water) and incubated for 5 min at 25°C, then 10% Na_2CO_3 solution was added. After incubation at 25°C for 90 min, the absorbance was measured at 730 nm. All tests were performed six times. The phenolic content was evaluated from a gallic acid standard curve.

(Ⅵ) Protective effect of DT and EF on CCl_4-Induced acute hepatotoxicity

1. Preparation of CCl_4-Induced acute hepatotoxicity

The animals were randomly divided into nine groups with each consisting of 12 mice. Group I served as normal control. For inducing hepatotoxicity (*in vivo*), animals of Groups II- IX were administered orally 1 mL/kg body weight of carbon tetrachloride (20% CCl_4 in olive oil). Group II served as negative control. Group III served as positive control and was given silymarin (200 mg/kg, p.o.). Groups IV–VI was administered orally the DT extract at doses of 100, 500, and 1000 mg/kg, respectively. Groups VII–IX was administered orally the EF extract at doses of 100, 500, and 1000 mg/kg, respectively. One hour after administration of the experimental drugs, CCl_4 (0.2%, 1ml/kg) was injected intraperitoneally into each group of mice except the normal group. Normal group mice received a comparable volume of olive oil (i.p.). Twenty-four hours after CCl_4 injection, mice were sacrificed by cervical dislocation. Blood was collected into heparinized tubes (50 U/mL). One gram liver tissue was added with 1 ml of ice normal saline (pH = 7.0). The IKA-WEAR homogenizer (RW 20 DZM, Staufen, Germany) was used to homogenate the tissue (200 rpm and amplitude 10 times). The homogenate solution was centrifuged at 12000 rpm for 10 min at 4°C. The supernatant used for assay of the marker enzymes (GP_X, GST, GR, SOD and catalase), GSH, MDA, and protein estimation was immediately stored at -80°C until analysis. An extra sample of liver was excised and fixed in 10% formalin solution for histopathologic analysis.

2. Measurement of serum ALT, AST

Liver damage was assessed by the estimation of serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using commercially available test kits from by Randox Laboratories Ltd. (UK). The results were expressed as units/liter (IU/L).

3. Measurement of SOD, GPx, and GR activities in liver homogenate

Liver homogenates were prepared in cold Tris-HCl (5 mmol/L containing 2 mmol/L EDTA, pH 7.4) using a homogenizer. The unbroken cells and cell debris were removed by centrifugation at 10000 rpm for 10 min at 4°C. The supernatant was used immediately for the assays of SOD, GPx and GR. All of these enzymes were determined following the instructions on the Randox Laboratories Ltd kit.

4. Measurement of catalase (CAT) in liver homogenate

Catalase (CAT) activity was measured by the method of Aebi[21]. The supernatant (0.1 mL) was added to cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). Reaction was started by addition of 1.0 mL of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm. Activity of catalase was expressed as U/mg of protein.

5. Measurement of GSH content in liver homogenate

GSH content was determined according to the method of Ellman[22] with slight modification. The homogenate solution was mixed with TCA solution, and placed in ice box for 5 min and then centrifuged for 30 mins. The 25 µL upper layer, 600 µL Saline solution (pH = 7) and 125 µL 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) solution were mixed well. After 5 min, the absorbance was measured at 412 nm. The GSH content was calculated according GSH standard curve.

6. Measurement of GST activity in liver homogenate

Glutathione-s-transferase activity was estimated by the method of Habig *et al.*[23]. The reaction mixture consisted of 2.75 mL of sodium phosphate buffer (0.1 M; pH 7.4), 0.1 mL reduced glutathione (1 mM), 0.1 mL supernatant in a total volume of 3.0 mL. The changes in the absorbance were recorded at 340 nm and enzymes activity was calculated as nanomoles of 1-chloro-2,4-dinitro benzene (CDNB) conjugate formed/min/mg protein.

7. Measurement of lipid peroxidation in liver homogenate

The quantitative measurement of lipid peroxidation was done by measuring the concentration of TBARS in liver using the method of Tatum *et al*[24]. The supernatant of liver tissue homogenate (1 ml) was mixed with 1 ml of 7.5% (w/v) cold trichloroacetic acid

(TCA) to precipitate proteins and then centrifuged at 1500 rpm. The supernatant was reacted with 1 ml of 0.8% (w/v) TBA in a boiling water bath for 45 min. After cooling, the lipid peroxidation product (MDA) was assayed according to an improved thiobarbituric acid reactive substances (TBARS) fluorometric method after excitation at 555 nm and emission at 515 nm using 1,1,3,3-tetraethoxypropane (TEP) as the standard. The results were expressed as IU of TBARS/mg of protein.

8. Measurement of total protein content in liver homogenate

Protein concentration was estimated according to the method of Lowry *et al*[25], using bovine serum albumin as a standard.

9. Assessment of liver damage

Liver tissues were placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. The fixed tissues were processed routinely, embedded in paraffin, sectioned, deparaffinized, and rehydrated using the standard techniques. The extent of CCl_4-induced necrosis was evaluated by assessing the morphological changes in the liver sections (five micron sections) stained with hematoxylin and eosin (H&E), using standard techniques.

10. Statistical Analysis

All results are expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA followed by Scheffe's test. Differences between experimental groups were considered statistically significant when p value < 0.05 was set as the threshold of statistical significance.

Results

I. Anti-oxidative activity of DT_{EtOH} and EF_{EtOH}

The anti-oxidative activity of DT_{EtOH} and EF_{EtOH} has been determined by three different test systems namely DPPH scavenging, reducing power and ferrous metal-chelating capacities assays (Fig 1). As shown in Fig 1A-1C, the DT_{EtOH} and EF_{EtOH} possessed DPPH scavenging, reducing power and metal ion chelating capacities.

II. Total flavonoids and polyphenols contents

As shown in Table 1, DT_{EtOH} and EF_{EtOH} contained flavonoid (19.6 ± 0.4, 30.3 ± 0.6 mg gallic acid/g) and polyphenols (8.9 ± 0.6, 11.6 ± 0.5 mg quercetin/g).

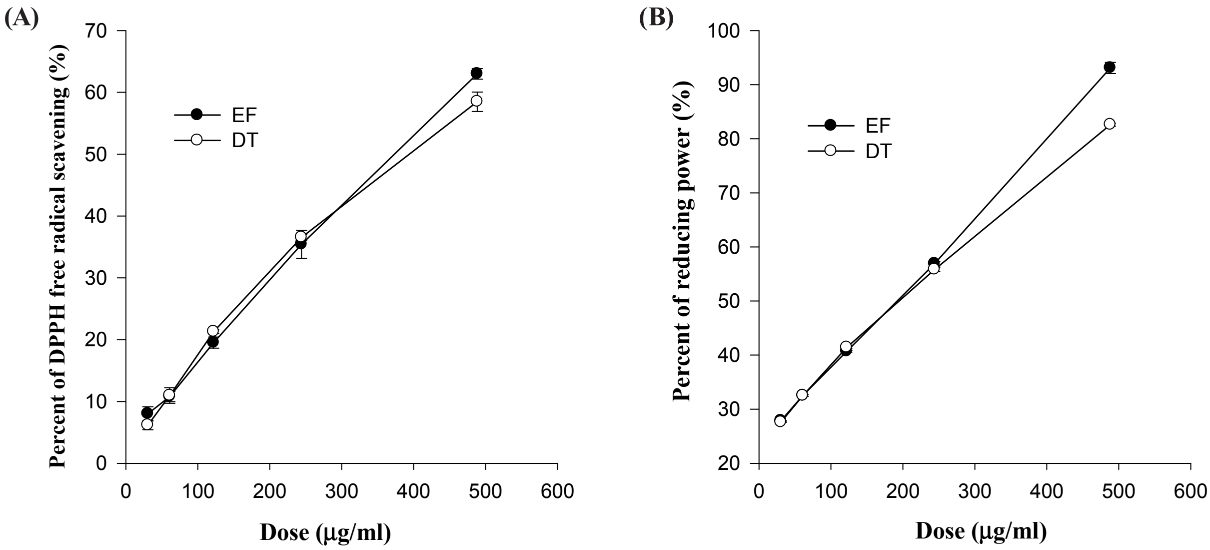
III. Protective effect of DT and EF on CCl_4-induced acute hepatotoxicity

As shown in Fig 2, mice treated with single dose of CCl_4 developed a significant hepatic damage and oxidative stress, which was observed from a substantial increase in the activities of sALT and sAST. The levels of sALT and sAST were reduced to the respective normal values by DT_{EtOH} and EF_{EtOH} at all treated doses.

IV. Changes of SOD, CAT, GPx, GR, GST activities and GSH level in liver homogenate

The SOD and CAT activities were brought to near normal after pretreatment with the DT_{EtOH} and EF_{EtOH} in CCl_4-treated mice (Fig 3, Fig 4) evidently show the antioxidant property of the DT_{EtOH} and EF_{EtOH} against oxygen free radicals.

Reduction in liver GSH and decrease in GPx activity in CCl_4-treated mice as observed in this study



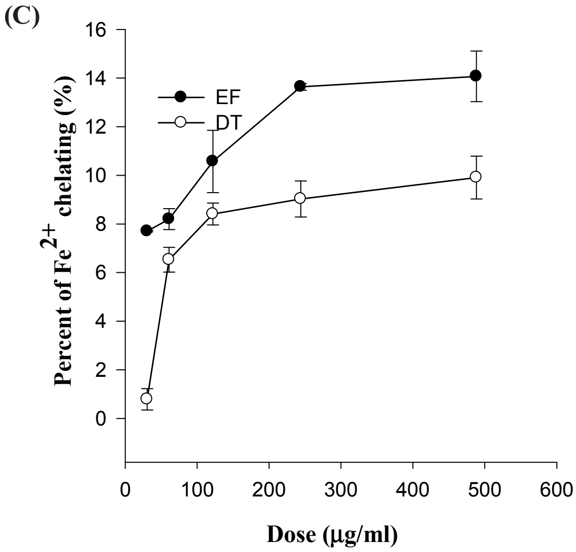


Fig. 1. The anti-oxidative activities of DT_{EtOH} and EL_{EtOH} were determined by (A) DPPH free radical scavenging test, (B) Reducing power test and (C) Ferrous metal-chelating capacities test. Data are expressed as means ± S.E.M. (n= 3).

Table 1. Total polyphenols and flavonoids contents of EF_{EtOH} and DT_{EtOH}.

Groups	Polyphenols (mg gallic acid/g)	Flavonoids (mg quercetin/g)
EF_{EtOH}	30.3 ± 0.6	11.6 ± 0.5
DT_{EtOH}	19.6 ± 0.4	8.9 ± 0.6

Data are expressed as mean ± SD (n = 4)

(Fig 5, 6) indicates the damage to the hepatic cells. Administration of DT_{EtOH} and EF_{EtOH} promoted the conversion of GSSG (oxidized glutathione) into GSH by the reactivation of hepatic glutathione reductase enzyme in CCl_4-treated mice.

GST activity was significantly reduced in CCl_4-treated mice and upward reversal was observed after treatment with the DT_{EtOH} and EF_{EtOH} at two different doses (Fig 7). In CCl_4 treated mice, the activity of GR is markedly decreased. An increase in GR activity indicates that the DT_{EtOH} and EF_{EtOH} protect the liver tissue from oxidative damage (Fig 8).

V. Changes of MDA level in liver homogenate

In CCl_4 treated mice, the MDA level is markedly increased. Treatment with DT_{EtOH} and EF_{EtOH} at three different doses (0.1, 0.5, 1g/kg) and standard drug, silymarin (200 mg/kg) were seen to decrease MDA value obviously when compared with CCl_4 treated mice (Fig 9).

VI. Assessment of liver damage

In normal animals group, liver sections showed normal hepatic cells with well cytoplasm, prominent nucleus and nucleolus and central vein (Fig 10-A).

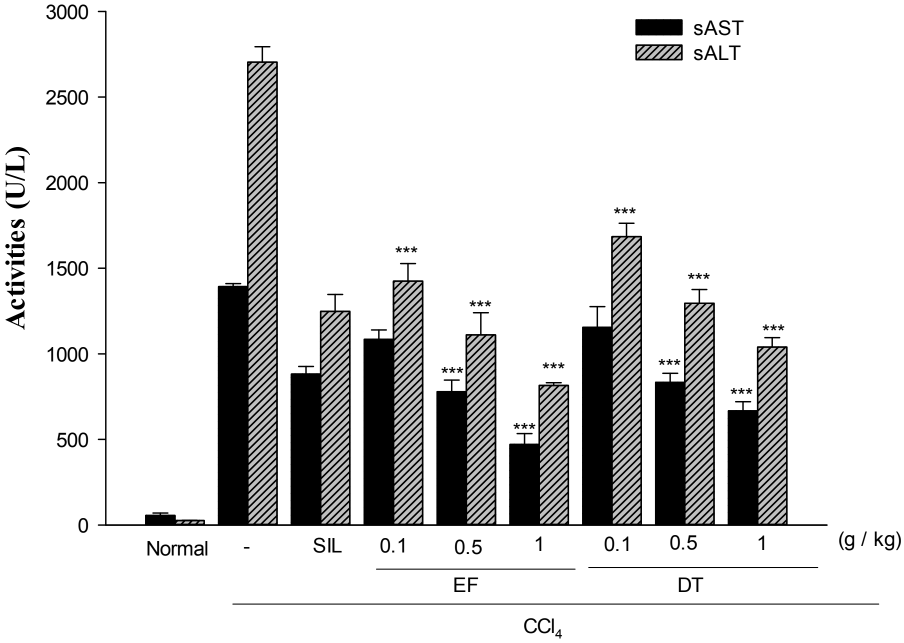


Fig. 2. Effect of DT_{EtOH} and EL_{EtOH} on sALT and sAST activities in CCl_4–induced acute liver damage. Each value represented as mean ± S.E.M. (n=10). ***p<0.001 as compared with the CCl_4 group.

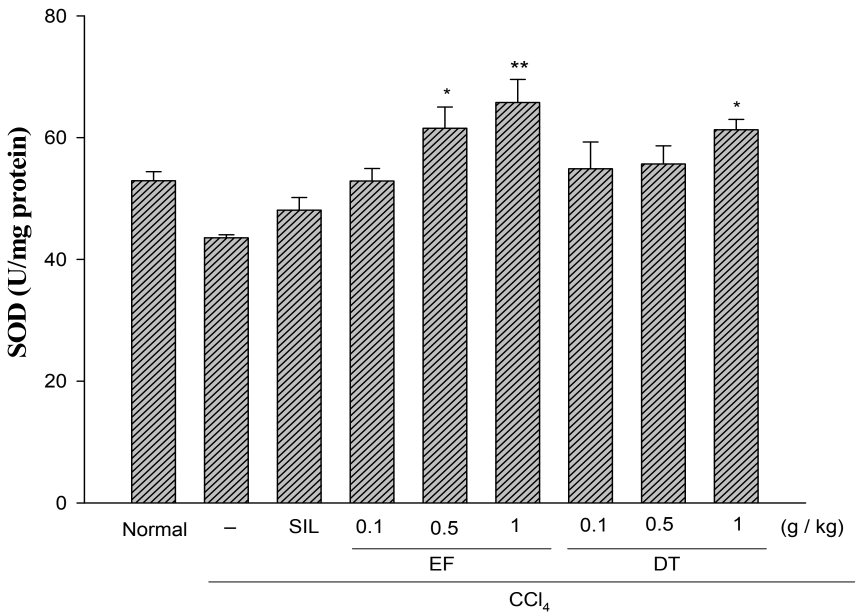


Fig. 3. Effect of DT_{EtOH} and EL_{EtOH} on liver SOD activities in CCl_4–induced acute liver damage. Each value represented as mean ± S.E.M.. *p<0.05, **p<0.01 as compared with the CCl_4 group.

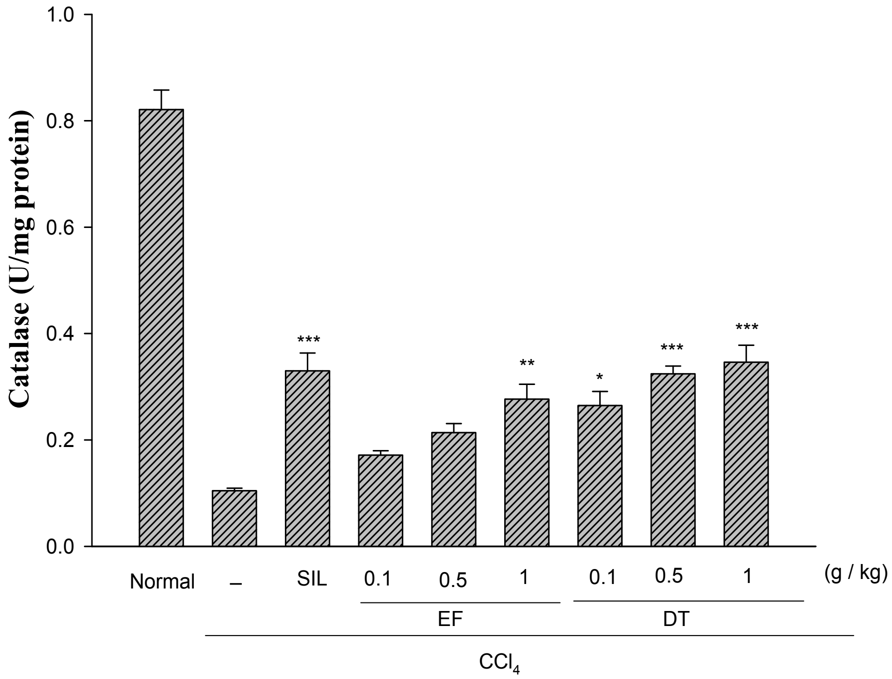


Fig. 4. Effect of DT_{EtOH} and EL_{EtOH} on liver catalase (CAT) activities in CCl_4–induced acute liver damage. Each value represented as mean ± S.E.M.. *p<0.05, **p<0.01, ***p<0.001 as compared with the CCl_4 group.

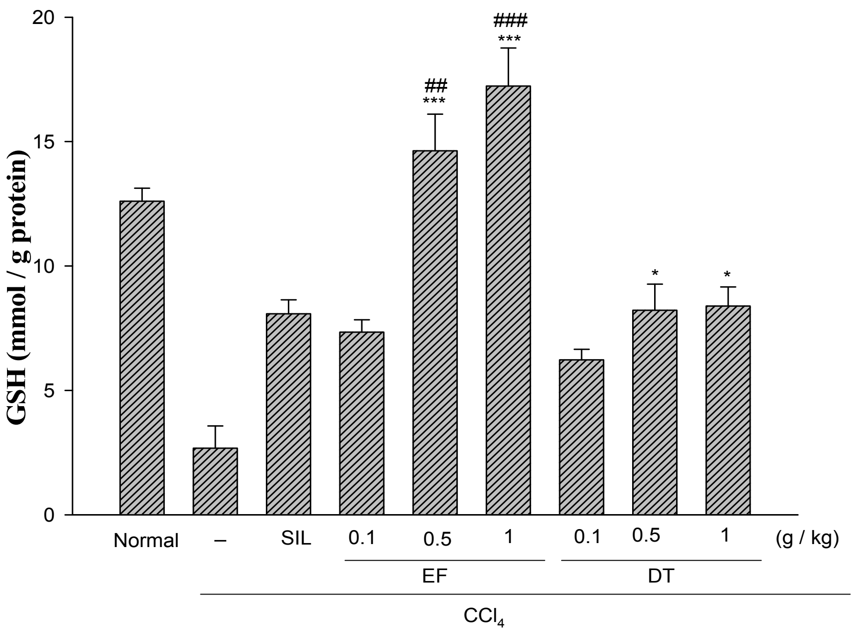


Fig. 5. Effect of DT_{EtOH} and EL_{EtOH} on liver GSH level in CCl_4–induced acute liver damage. Each value represented as mean ± S.E.M.. *p<0.05, ***p<0.001 as compared with the CCl_4 group. ## p<0.05, ### p<0.001 as compared with the DT_{EtOH} group equal dose.

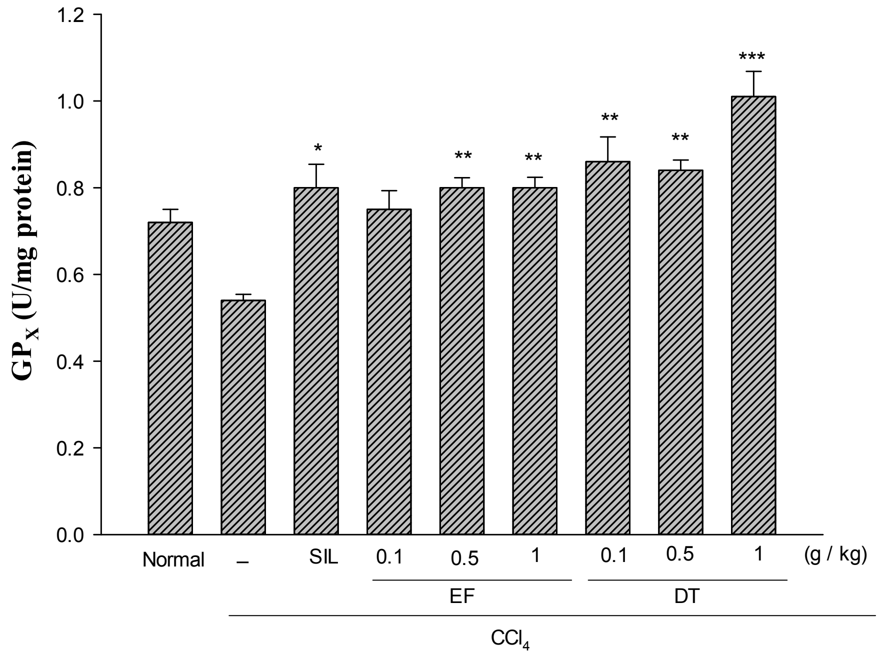


Fig. 6. Effect of DT_{EtOH} and EL_{EtOH} on liver GPx activities in CCl_4–induced acute liver damage. Each value represented as mean ± S.E.M.. *p<0.05, **p<0.01, ***p<0.001 as compared with the CCl_4 group.

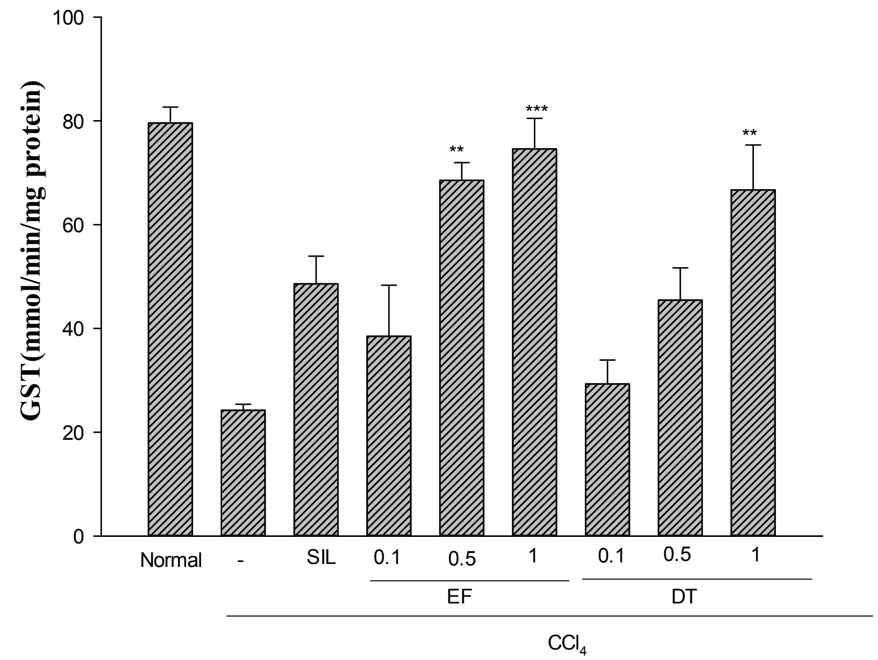


Fig. 7. Effect of DT_{EtOH} and EL_{EtOH} on liver GST activities in CCl_4–induced acute liver damage. Each value represented as mean ± S.E.M. (n=10). **p<0.01, ***p<0.001 as compared with the CCl_4 group.

The mice liver treatment with CCl_4 revealed moderate ballooning degeneration, serious necrosis, and mild inflammatory cell infiltration of hepatocytes (Fig 10-B). Compared with the lesions observed in the CCl_4 group, the lesions treated with silymarin have moderated improvement (Fig 10-C). The groups treated

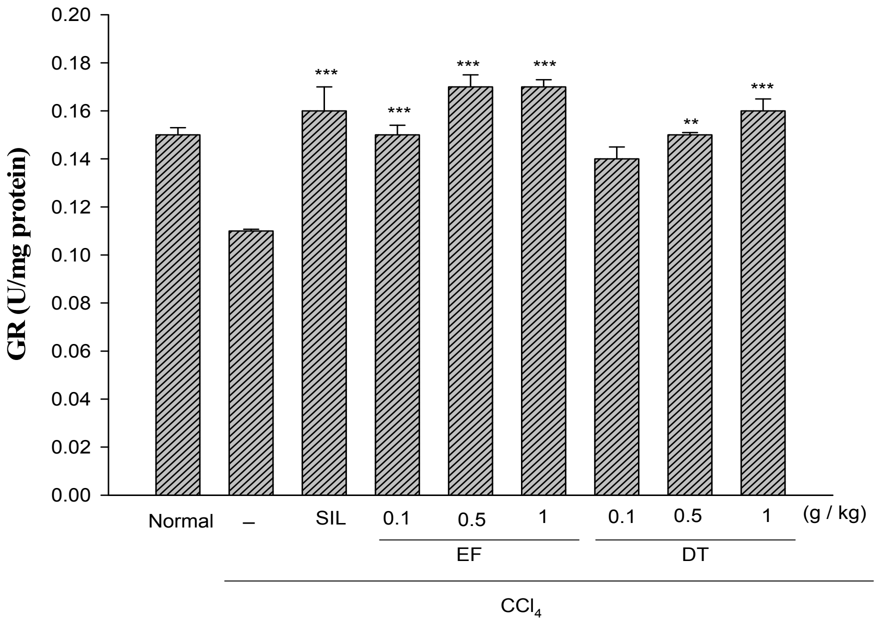


Fig. 8. Effect of DT_{EtOH} and EL_{EtOH} on liver GR activities in CCl_4–induced acute liver damage. Each value represented as mean ± S.E.M.. **p<0.01, ***p<0.001 as compared with the CCl_4 group.

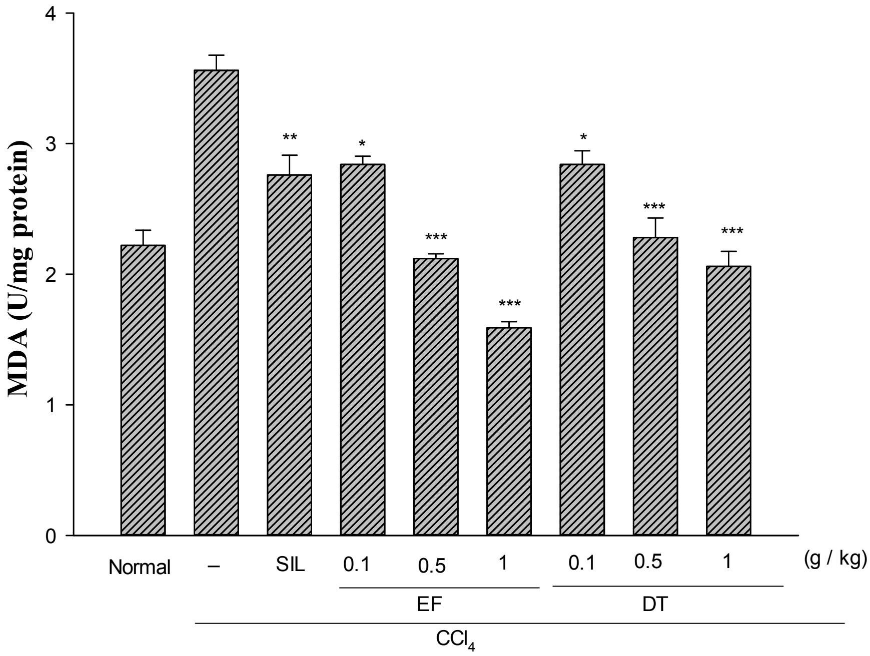


Fig. 9. Effect of DT_{EtOH} and EL_{EtOH} on liver MDA content in CCl_4–induced acute liver damage. Each value represented as mean ± S.E.M.. *p <0.05, **p<0.01, ***p<0.001 as compared with the CCl_4 group.

with DT_{EtOH} and EF_{EtOH} at 0.1, 0.5, and 1 g/kg showed mild to light diffused necrosis of hepatocytes, mild inflammatory cell infiltration, and trace ballooning degeneration, respectively (Fig 10-D–I).

Discussion

Antioxidant activity of the two "Shi-Hu" has been determined by three different test systems. DPPH

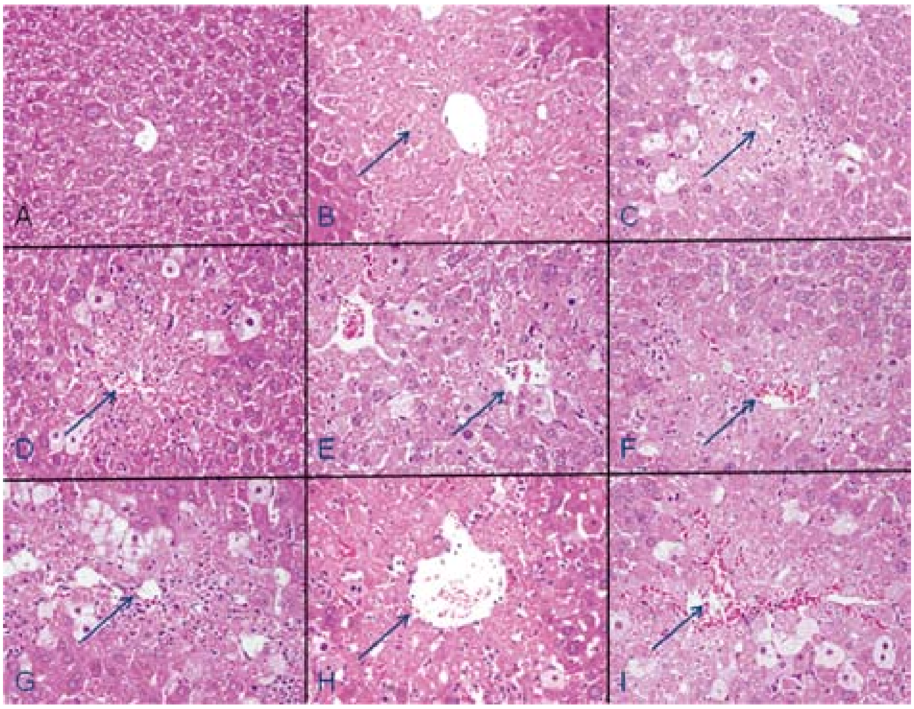


Fig. 10. Effect of DT_{EtOH} and EL_{EtOH} on hepatic morphological analysis in CCl_4-toxicated mice. Livers were sectioned and stained with hematoxylin eosin by standard techniques (400X). (A) Normal control, (B) CCl_4 control, (C) Silymarin (200 mg/kg) + CCl_4, (D) *EF* (0.1g/kg) + CCl_4, (E) *EF* (0.5g/kg) + CCl_4, (F) *EF* (1.0 g/kg) + CCl_4, (G) *DT* (0.1g/kg) + CCl_4, (H) *DT* (0.5g/kg) + CCl_4, (I) *DT* (1.0 g/kg) + CCl_4. Arrow line indicates the central vein.

scavenging test can simulate a condition of free radical decreased by degree of discoloration when treatment with drug, and evaluate antioxidative activity for drug[16]. Reducing power is capacity for reducing peroxide generation to terminate free radical reaction, and is good test for evaluated antioxidative activity for drug[26]. Metal iron can stimulate lipid peroxidation by the Fenton reaction and decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can perpetuate the chain reaction[27]. If the metal ion was chelated by the test drugs, the Fenton reaction is terminated and free radicals will decrease. The DT_{EtOH} and EF_{EtOH} possessed DPPH scavenging, reducing power and metal ion chelating capacities. In the previous study, Dendrobium species contain flavonoids and polyphenols[13, 28]. Flavonoids and polyphenols possess antioxidative activity and capacity of scavenging free radical[29]. Therefore, flavonoids and polyphenols may be the active compositions of DT_{EtOH} and EF_{EtOH}.

Carbon tetrachloride (CCl_4) is an extensively used industrial solvent, and it is the best toxic-induced drug in animal model of hepatotoxicity from free radical damage[30]. In this study, mice treated with single dose of CCl_4 developed a significant hepatic damage and oxidative stress, which was observed from a substantial increase in the activities of sALT and sAST. This is indicative of cellular leakage and loss of functional integrity of cell membrane in liver[31]. Reduction in the levels of sALT and sAST towards the respective normal values by DT_{EtOH} and EF_{EtOH} at all treated doses (100 to 1000 mg/kg) is an indication of the stabilization of plasma membranes as well as repair of hepatic tissue damage caused by CCl_4. This effect is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes[32]. This indicates the anti-lipid peroxidation and/or adaptive nature of the systems as brought about by DT_{EtOH} and EF_{EtOH} against the damaging effects of free radicals produced by CCl_4. During hepatic injury, superoxide radicals generate at the site of damage and modulate SOD and CAT, resulting in the loss of activity and accumulation of superoxide radical, which damages liver. Decreased CAT activity is linked up to exhaustion of the enzyme as a result of oxidative stress caused by CCl_4. The SOD and CAT activities were brought to near normal after pretreatment with the DT_{EtOH} and EF_{EtOH} in CCl_4-treated mice evidently shows the antioxidant property of the DT and EF extracts against oxygen free radicals.

Reduced glutathione (GSH) constitutes the first line of defense against free radicals. Reduction in liver GSH and decrease in GPx activity in CCl_4-treated mice as observed in this study indicates the damage to the hepatic cells. Administration of DT_{EtOH} and EF_{EtOH} promoted the conversion of GSSG (oxidized glutathione) into GSH by the reactivation of hepatic glutathione reductase enzyme in CCl_4-treated mice. The availability of sufficient amount of GSH thus increased the detoxification of active metabolites of CCl_4 through the involvement of GPx. But the restoration of GSH level after the treatment of DT_{EtOH} and EF_{EtOH} to such CCl_4 treated mice is account for the protective efficacy of the extracts. The reduced glutathione level of DT_{EtOH} and EF_{EtOH} treated groups are in accordance with the report of Chao[33]. GPx activity was significantly reduced after CCl_4 treatment when compared to control, which indicates the damage to the hepatic cells[34]. The reversal of the GPx activity after pretreatment with DT_{EtOH} and EF_{EtOH} is due to antioxidant activity by scavenging/detoxifying the endogenous metabolic peroxides generated after CCl_4 injury in the liver tissue. GST plays a physiological role in initiating the detoxification of potential alkylating agents. Chemicals like chloroform and CCl_4 alter the hepatic GST activity[35]. GST activity was significantly reduced in CCl_4-treated mice and upward reversal was observed after treatment with the DT_{EtOH} and EF_{EtOH} at two different doses. This may be attributed to a direct action of extracts on the hepatic GST activation. GSSG is reduced to GSH by glutathione reductase, which is NADPH-dependent. It plays a role in maintain adequate amounts of GSH. Accordingly, the reduction of GR results in decreasing GSH[36]. In CCl_4 treated mice, the activity of GR is markedly decreased. An increase in GR activity indicates that the DT and EF protect the liver tissue from oxidative damage.

MDA is produced when peroxidation of biological membrane polyunsaturated fatty acid[37]. The increase in MDA level reflects peroxidation leading to tissue damage and failure of the antioxidant defense mechanisms to prevent the formation of excessive free radicals. MDA and GSH content can be marks of oxidative stress state and confirm improvement of liver injury. In the present study, MDA level was significantly increased in CCl_4 treated mice when compared to control mice. Treatment with DT_{EtOH} and EF_{EtOH} at three different doses (0.1, 0.5, 1g/kg) and standard drug, silymarin (200 mg/kg) were seen to decrease MDA level obviously when compared with CCl_4 treated mice. Glutathione (GSH) of liver homogenates significantly decreased in CCl_4 treated mice when compared to control mice. Treatment with DT_{EtOH} and EF_{EtOH} at three different doses (0.1, 0.5, 1g/kg) and standard drug, silymarin (200 mg/kg) were seen to increase GSH value obviously when compared with CCl_4 treated mice. It expressed DT_{EtOH} and EF_{EtOH} can promote the antioxidative state to prevent oxidative stress damage.

The mice liver treatment with CCl_4 revealed moderate ballooning degeneration, serious necrosis, and mild inflammatory cell infiltration of hepatocytes. The lesions treated with silymarin have moderated improvement. The groups treated with DT_{EtOH} and EF_{EtOH} at 0.1, 0.5, and 1 g/kg showed mild to light diffused necrosis of hepatocytes, mild inflammatory cell infiltration, and trace ballooning degeneration. In the present result, necrosis improvement of hepatocytes were observed obviously with EF_{EtOH} dose increase and have more better effect than equal dose of DT_{EtOH}. The date has good correlation with the results of the sALT and sAST activities and MDA level from hepatic lipid peroxidation. On the other hand, EF is cheaper than DT and silymarin in the market.

In conclusion, the EF_{EtOH} and DT_{EtOH} possessed hepatoprotective effect on CCl_4-induced acute liver injury as well as oxidative stress, resulting in reducing MDA level and improving serum biochemical parameters such as sALT and sAST. The activity of EF_{EtOH} at the doses of 500 mg/kg was comparable to the standard drug, silymarin (200 mg/kg). EF could be used as the resources of Shi-Hu. Therefore, it is worth to develop the EF that is benefited for liver disease.

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黃花石斛及有瓜石斛抗氧化活性及抗四氯化碳誘導急性肝損傷

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本研究目的主要探討台灣栽培的黃花石斛及有瓜石斛市售品的抗氧化活性及對四氯化碳誘導的急性肝臟傷害的保護效果，以總多酚和類黃酮含量試驗來評估四種石斛活性成分含量，再以 DPPH 自由基清除試驗和還原力試驗以及亞鐵螯合能力試驗等三種抗氧化能力試驗來評估二種石斛的抗氧化活性，再探討其對四氯化碳誘導的急性肝損傷的保護效果，測定 catalase, SOD, GPX, GSH, GR, GST 及 MDA，最後觀察其肝臟病理切片以探討石斛保護四氯化碳誘導急性肝損傷之機轉。

實驗結果顯示，有瓜石斛及黃花石斛均含有多酚及類黃酮，在體外抗氧化實驗中，具有抗氧化效果。有瓜石斛及黃花石斛對四氯化碳所引起血清 AST 及 ALT 活性升高有顯著降低，並減少 MDA 濃度，此結果與病理切片結果一致。有瓜石斛及黃花石斛可以提升因四氯化碳所減少抗氧化酵素活性和 GSH 濃度。綜合實驗結果，顯示有瓜石斛及黃花石斛具有抗氧化活性及保護四氯化碳誘導的急性肝臟損傷，其保護機制可能經由提升自由基清除酵素的活性及 GSH 濃度來達到保護效果。

關鍵字：黃花石斛、有瓜石斛、抗氧化活性、急性肝損傷、丙二醛

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