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Hepatoprotective effects of eburicoic acid and dehydroeburicoic acid from *Antrodia camphorata* in a mouse model of acute hepatic injury

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ABSTRACT

The hepatoprotective effects of eburicoic acid (**TR1**) and dehydroeburicoic acid (**TR2**) from *Antrodia camphorata* (AC) against carbon tetrachloride (CCl_4)-induced liver damage were investigated in mice. **TR1** and **TR2** was administered intraperitoneally (*i.p.*) for 7 days prior to the administration of CCl_4. Pretreatment with **TR1** and **TR2** prevented the elevation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and liver lipid peroxides in CCl_4-treated mice. The activities of antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx)], nitric oxide (NO) production, and tumour necrosis factor-alpha (TNF-α) were decreased after the treatment with **TR1** and **TR2** in CCl_4-treated mice. Western blotting revealed that **TR1** and **TR2** significantly decreased inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions and increased the expression of cytochrome P4502E1 (CYP2E1) in CCl_4-treated mice. Therefore, we speculate that **TR1** and **TR2** protect the liver from CCl_4-induced hepatic damage *via* antioxidant and anti-inflammatory mechanisms.

1. Introduction

Liver diseases are a major problem throughout the world. The liver plays a key role in the metabolism, detoxification, and secretionary functions of the body. A wide variety of viruses, drugs, and toxic chemicals can cause liver injury by means of their direct toxicity and/or endogenous toxic metabolic products (Huang et al., 2012).

Carbon tetrachloride (CCl_4) is commonly used as a chemical inducer of experimental liver injury (Huang et al., 2011). CCl_4 is responsible for oxidative stress and lipid peroxidation through cytochrome P4502E1 (CYP2E1)-mediated generation of highly reactive radicals, leading to eventual damage characterised by hepatocellular necrosis (Yu, Qian, & Lu, 2011). The process is followed by the release of inflammatory mediators including TNF-α and NO from the activated hepatic macrophages, which are believed to potentiate the CCl_4-induced hepatic injury (Huang et al., 2012).

Antrodia camphorata (AC; Polyporaceae, Aphyllophorales) is a parasitic microorganism found on the wall of the inner cavity of *Cinnamomum kanehirai* Hay. The major bioactive compounds of AC are currently identified as fatty acids, polysaccharides, triterpenoids, steroids, benzenoids, sesquiterpenes, steroids, and maleic/succinic acid derivatives (Shao et al., 2008). The pharmacological properties of AC include hepatoprotective properties against acute alcohol-, carbon tetrachloride (CCl_4)-, lipopolysaccharide-, and hepatitis B virus-induced injuries, as well as retardation of liver fibrosis, anti-invasion, and anti-metastasis of liver cancer cells (Hseu, Chen, Chen, Liao, & Yang, 2008). AC mycelium is often used to evaluate its hepatoprotective effects; meanwhile, its hepatoprotective effect is attributed to its antioxidant capacity. Recently, our previous paper indicated that antrosterol from AC submerged whole broth has enhanced antioxidant activity and can inhibit lipid peroxidation in liver (Huang et al., 2011). Eburicoic acid (**TR1**) was isolated from *Laetiporus sulphureus* (León, Quintana, Rivera, Estévez, & Bermejo, 2004), and AC (Chen et al., 2006). Dehydroeburicoic

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acid (**TR2**) was isolated from *Poria cocos* (Lee et al., 2009) and AC (Majid et al., 2009). **TR1** and **TR2** were found to inhibit the proliferation of human HL-60 myeloid leukaemia cells (León, Quintana, Rivera, Estévez, & Bermejo, 2004; Du et al., 2012). **TR2** inhibited human 5-hydroxytryptamine 3A (5-HT (3A)) receptor channel activity (Lee et al., 2009) and induces calcium- and calpain-dependent necrosis in human U87MG glioblastomas (Deng, Chen, Jow, Hsueh, & Jeng, 2009). However, little information is available on the protective effects against CCl_4-induced hepatic toxicity effects of **TR1** and **TR2**. To our knowledge, this study is the first report that demonstrates the anti-inflammatory effects of **TR1** and **TR2** in liver protection and the blocking of iNOS, COX-2, and CYP2El-mediated CCl_4 bioactivities in CCl_4-induced liver injury model by AC components.

2. Material and methods

2.1. Chemicals

Carbon tetrachloride was purchased from Merck (Darmstadt, Germany). Silymarin, malondialdehyde, and other chemicals were purchased from Sigma Chemical Co. (Steinheim, Germany). Biochemical assay kits for measurement of ALT and AST contents were purchased from Randox Laboratories (Crumlin, UK). TNF-α was purchased from Biosource International Inc., (Camarillo, CA). Anti-iNOS, anti-COX-2, anti-CYP2E1, and anti-β-actin antibody (Abcam, Cambridge, MA) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, UK) were obtained as indicated. Poly-(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA).

2.2. Fungus material

The solid culture of fruiting body was provided and identified by Taiwan Leader Biotech Corp., New Taipei, Taiwan. A voucher specimen was deposited at Well Shine Biotechnology Development Co. Ltd.

2.3. Isolation and determination of the active compound

Freeze-dried powders of the solid culture of AC (3.0 kg) were extracted three times with methanol (12 L) at room temperature (4 day × 3). The methanol extract was evaporated in vacuo to give a brown residue, which was suspended in H_2O (1 L), and then partitioned with ethyl acetate (1 L × 3). The EtOAc fraction (200 g) was chromatographed on silica gel using mixtures of hexane and EtOAc of increasing polarity as eluents and further purified with HPLC. Eburicoic acid (**TR1**) and dehydroeburicoic acid (**TR2**) were isolated by HPLC on a Hibar® pre-packed column RT 250-10 and using a refractive index (RI) detector (Knauer RI detector 2400) with chloroform:ethyl acetate (7:1). The flow rate was 3 mL/min, and the injection volumes of samples were 100 μL. The yields of **TR1** and **TR2** obtained were about 0.1% and 0.2% (w/w). The purity of **TR1** and **TR2** were higher than 99% (Chen et al., 2006; Majid et al., 2009) (Fig. 1A).

TR1: ^{1}H NMR (300 MHz, pyridine-d_5): δ 3.41 (1H, br t, J = 7.6 Hz, H-3), 1.00 (3H, s, H-18), 1.06 (3H, s, H-19), 2.63 (1H, td, J = 2.4, 10.6 Hz, H-20), 2.27 (1H, m, H-25), 1.01 (6H, d, J = 7.6 Hz, H-26 and H-27), 4.87 (1H, br s, H-28a), 4.91 (1H, br s, H-28b), 1.05 (3H, s, H-29), 1.22 (3H, s, H-30), 1.00 (3H, s, H-31).

TR2: ^{1}H NMR (300 MHz, pyridine-d_5): δ 1.90 (2H, m, H-2), 3.43 (1H, t, J = 7.5 Hz, H-3), 1.26 (1H, H-5), 2.16 (2H, H-6), 5.61 (1H, br s, H-7), 5.36 (1H, d, J = 5.1 Hz, H-11), 2.50 (1H, H-12α), 2.33 (1H, H-12β), 0.99 (3H, s, H-18), 1.19 (3H, s, H-19), 2.64 (1H, td, J = 11.0, 3.0 Hz, H-20), 2.29 (1H, H-25), 1.02 (3H, d, J = 3.0 Hz, H-26 or H-27), 1.00 (6H, d, J = 7.5 Hz, H-27 and H-26), 4.88 (1H, br s, H-28α), 4.92 (1H, br s, H-28β), 1.11 (3H, s, H-29), 1.05 (6H, s, H-30, 31).

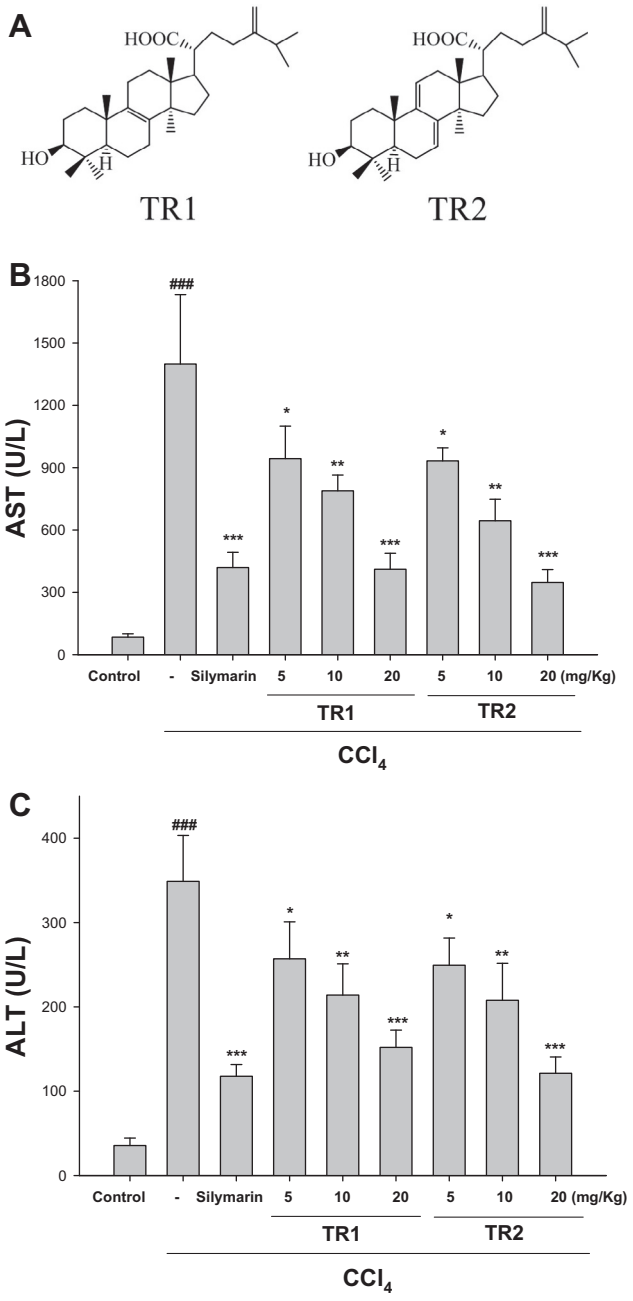

Fig. 1. Chemical structures of **TR1** and **TR2** (A) and the effect of **TR1** and **TR2** on the activities of serum ALT (B) and AST (C) in CCl_4-injected mice. The values are reported as the mean ± S.E.M. of six mice per group. $^{###}p < 0.001$ compared with control; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the CCl_4 group.

2.4. Animal treatment

6–8 weeks male imprinting control region (ICR) mice were obtained from the BioLASCO Taiwan Co., Ltd (Taipei, Taiwan). The animals were kept in plexiglass cages at a constant temperature of 22 ± 1 °C, and relative humidity of 55 ± 5% with 12 h dark–light cycle for at least 2 week before the experiment. They were given food and water *ad libitum*. Animal studies were conducted according to the regulations of the Instituted Animal Ethics Committee

and the protocol was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals. Mice were randomly divided into six groups of six animals each ($n = 6$). Mice in the normal control (0.2 mL/10 g body weight) and negative control (0.2 mL/10 g body weight) groups were administered with distilled water. The positive control group was administered with silymarin (200 mg/kg in 1% carboxymethyl cellulose (about 10 mL, *i.p.*)) once daily for 7 days. In the three experimental groups, the mice were pretreated with **TR1** and **TR2** (5, 10, and 20 mg/kg in 1% carboxymethyl cellulose, *i.p.*) once daily for seven consecutive days. One hour after the last treatment, all the mice, except for those in the normal control, were treated with CCl_4 (1.5 mL/kg in olive oil, 20%, *i.p.*). 24 h after the CCl_4 treatment, animals were anaesthetised with ethyl ether and blood samples were collected through their carotid arteries. The mortality rate and body weight were recorded every day.

2.5. Assessment of liver functions

The blood was centrifuged at 1700*g* (Beckman GS-6R, Germany) at 4 °C for 30 min to separate serum. ALT and AST were analysed. Liver tissues collected from the animals were fixed in 10% formalin for histopathological studies. Also, liver tissue was kept at −80 °C for further analysis of their enzyme levels. The biochemical parameters were analysed by using clinical test kits (Roche Cobas Mira Plus, Germany).

2.6. Histopathological examination

Small pieces of liver, the anterior portion of the left lateral lobe of the liver, fixed in 10% buffered formalin were processed for embedding in paraffin. Sections of 4–5 μm were cut and stained with haematoxylin and eosin, and then examined for histopathological changes under the microscope (Nikon, ECLIPSE, TS100, Japan). Images were taken with a digital camera (NIS-Elements D 2.30, SP4, Build 387) at original magnification of 200×.

2.7. Antioxidant enzyme activity measurements

The following biochemical parameters were analysed to check the hepatoprotective activity of **TR1** and **TR2** by the methods given below.

Total superoxide dismutase (SOD) activity was determined by the inhibition of cytochrome *c* reduction (Flohe & Otting, 1984). The reduction of cytochrome *c* was mediated by superoxide anions generated by the xanthine/xanthine oxidase system and monitored at 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by 50%.

Total catalase (CAT) activity estimation was based on that of Aebi (1984). In brief, the reduction of 10 mM H_2O_2 in 20 mM of phosphate buffer (pH 7) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a molar absorption coefficient, and the enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute.

Total GPx activity in cytosol was determined as previously reported (Paglia & Valentine, 1967). The enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2), and the absorbance at 340 nm was measured. The activity was evaluated from a calibration curve, and the enzyme activity was defined as nanomoles of NADPH oxidised per milligram protein per minute.

2.8. Measurement of hepatic GSH level

Hepatic GSH levels were determined by the method of Ellman with slight modification (Chang et al., 2011). Briefly, 720 μL of the liver homogenate in 200 mM Tris–HCl buffer (pH 7.2) were diluted to 1440 μL with the same buffer. Five percent TCA (160 μL) was added to it and mixed thoroughly. The samples were then centrifuged at 10,000*g* for 5 min at 4 °C. Supernatant (330 μL) was taken in a tube and 660 μL of Ellman's reagent (DTNB) solution were added to it. Finally the absorbance was measured at 405 nm. Protein content in each sample was determined by a bicinchoninic acid (BCA) protein assay kit (Pierce).

2.9. Lipid peroxidation intermediates

Thiobarbituric acid reactive substances (TBARS), in particular malondialdehyde (MDA), are the products of the oxidative degradation of polyunsaturated fatty acids. Lipid peroxidation was assayed by the measurement of MDA levels *via* absorbance at 535 nm on the basis of MDA reacting with thiobarbituric acid, as previously reported (Huang et al., 2011). Briefly, 0.4 mL of the treated cell or liver extract were mixed with 0.4 mL thiobarbituric acid reagent (consisting of 0.4% thiobarbituric acid (TBA) and 0.2% butylated hydroxytoluene (BHT). The reaction mixture was placed in water at 90 °C for 45 min, cooled, an equal volume of *n*-butanol was added, the mixture was centrifuged and the absorbance of the supernatant later was recorded at 535 nm. A standard curve was obtained with a known amount of 1,1,3,3-tetraethoxypropane (TEP), using the same assay procedure.

2.10. Measurement of serum TNF-α level by ELISA

Serum levels of TNF-α were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biosource International Inc., Camarillo, CA), according to the manufacturer's instruction. TNF-α was determined from a standard curve. The concentrations were expressed as pg/mL.

2.11. Measurement of nitric oxide/nitrite level

NO production was indirectly assessed by measuring the nitrite levels in serum determined by a calorimetric method based on the Griess reaction (Huang et al., 2010). Serum samples were diluted four times with distilled water and deproteinised by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at 10,000*g* for 5 min at room temperature, 100 μL supernatant were applied to a microtitre plate well, followed by 100 μL of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid). After 10 min of colour development at room temperature, the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices, Sunnyvale, CA). Using sodium nitrite to generate a standard curve, the concentration of nitrite was measured by absorbance at 540 nm.

2.12. Western Blot analysis

Liver tissue was homogenised in lysis buffer (0.6% NP-40, 150 mM NaCl, 10 mM HEPES (pH 7.9), 1 mM EDTA, and 0.5 mM PMSF) at 4 °C. Fifty micrograms of protein were fractionated on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA). Membranes were incubated with primary antibodies overnight at 4 °C using 1:1000 dilution of goat polyclonal anti-rabbit iNOS, COX-2, CYP2El, and β-actin antibodies. The membranes were washed three times and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc., Amersham, UK). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control, using Kodak Molecular Imaging Software (Version

4.0.5, Eastman Kodak Company, Rochester, NY) and represented in the relative intensities.

2.13. Statistical analysis

Data obtained from animal experiments were expressed as mean standard error (±S.E.M.). Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range tests). Statistical significance is expressed as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Effect of *TR1* and *TR2* on hepatotoxicity in CCl_4-treated mice

Several hepatic enzymes in serum such as AST and ALT were used as biochemical markers for early acute hepatic damage. The levels of AST and ALT were measured in the serum to evaluate hepatic tissue damage (Fig. 1B and C). CCl_4 administration resulted in significant ($p < 0.001$) rise in the levels of AST and ALT when compared with the control group. Intraperitoneal pre-administrations of **TR1** and **TR2** at three different doses (5, 10, and 20 mg/kg) significantly prevented the increased serum levels of ALT and AST. Silymarin (positive control) at a dose of 200 mg/kg also prevented the elevation of ALT and AST.

3.2. Histopathology of the liver

Based on the haematoxylin–eosin (H & E) stained tissue sections analysis, CCl_4 intoxication generated extensive changes in liver morphology, including steatosis, inflammation, hepatocytes ballooning, and necrosis (Fig. 2B). **TR1** and **TR2** pretreatment apparently alleviated liver damage, characterised by decreased necrotic zones and hepatocellular degeneration (Fig. 2D and E). This finding was consistent with the levels of the enzyme markers.

3.3. Effect of *TR1* and *TR2* on antioxidant enzymes activities in CCl_4-treated mouse liver

The hepatic antioxidant enzyme activities (SOD, CAT, and GPx) are shown in Fig. 3. The activities of SOD, CAT, and GPx were significantly decreased in CCl_4-treated mice, compared to the control. Mice pretreated with **TR1** and **TR2** at 20 mg/kg showed significant ($p < 0.001$) increase in SOD, CAT, and GPx when compared to the CCl_4 group (Fig. 3A–C). Silymarin-treated mice (the group of reference protective drug) also showed significant increase in SOD, CAT, and GPx when compared to CCl_4 treated mice.

3.4. Effect of *TR1* and *TR2* on lipid peroxidation in CCl_4-treated rat liver

As shown in Fig. 4A, hepatic levels of TBARS were assessed as an indicator of lipid peroxidation in the tissue. CCl_4 alone treated mice was observed a significant increase ($p < 0.001$) in tissue TBARS level. **TR1** and **TR2** at 5, 10, and 20 mg/kg significantly prevented the increase in TBARS level when compared to CCl_4 group. Moreover, silymarin also protected the liver from elevating TBARS levels and kept TBARS levels in normal values.

3.5. Effect of *TR1* and *TR2* on hepatic GSH levels in CCl_4-treated mice

The CCl_4-treatment caused significant ($p < 0.001$) decrease in the level of GSH in liver homogenate when compared with control group (Fig. 4B). The pretreatment of **TR1** and **TR2** at the dose of 5, 10, and 20 mg/kg resulted in significant increase of GSH content when compared to CCl_4 treated mice. Silymarin (200 mg/kg) treated mice also showed significant ($p < 0.001$) increase in GSH level in liver compared with CCl_4 group.

3.6. Effect of *TR1* and *TR2* on the serum level of TNF-α and NO in CCl_4-treated mice

As shown in Fig. 5A, the level of serum TNF-α was 37.45 ± 2.88 pg/mL in the control group. The CCl_4-treatment caused significant ($p < 0.001$) increase in the level of TNF-α in the serum when compared with the control group. The pretreatment of **TR1** and **TR2** at doses of 5, 10, and 20 mg/kg resulted in significant decrease of TNF-α level when compared to CCl_4-treated mice. Silymarin (200 mg/kg) treated mice also showed significant ($p < 0.001$) decrease in TNF-α level in serum compared with CCl_4-treated mice. As shown in Fig. 5B, the production of NO in mice serum was significantly increased in CCl_4-treated mice compared to

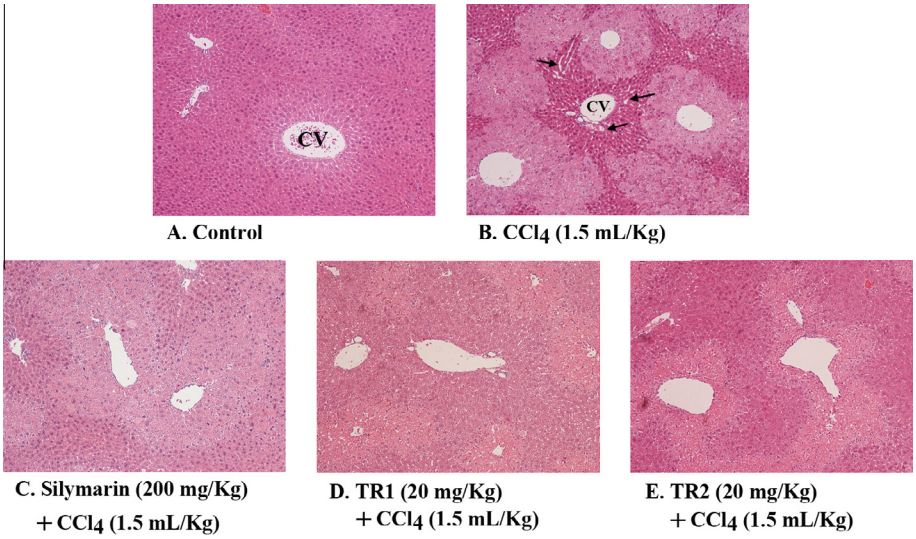

Fig. 2. Effect of **TR1** and **TR2** on CCl_4-induced liver damage. Sections were stained with haematoxylin–eosin (100×) and observed under light microscopy. (A) Control; (B) received CCl_4 (1.5 mL/kg); (C) silymarin (200 mg/kg) + CCl_4(1.5 mL/kg); (D) **TR1** (20 mg/kg) + CCl_4(1.5 mL/kg); (E) **TR2** (20 mg/kg) + CCl_4(1.5 mL/kg). Central vein (CV); arrows indicate fat vacuoles deposition.

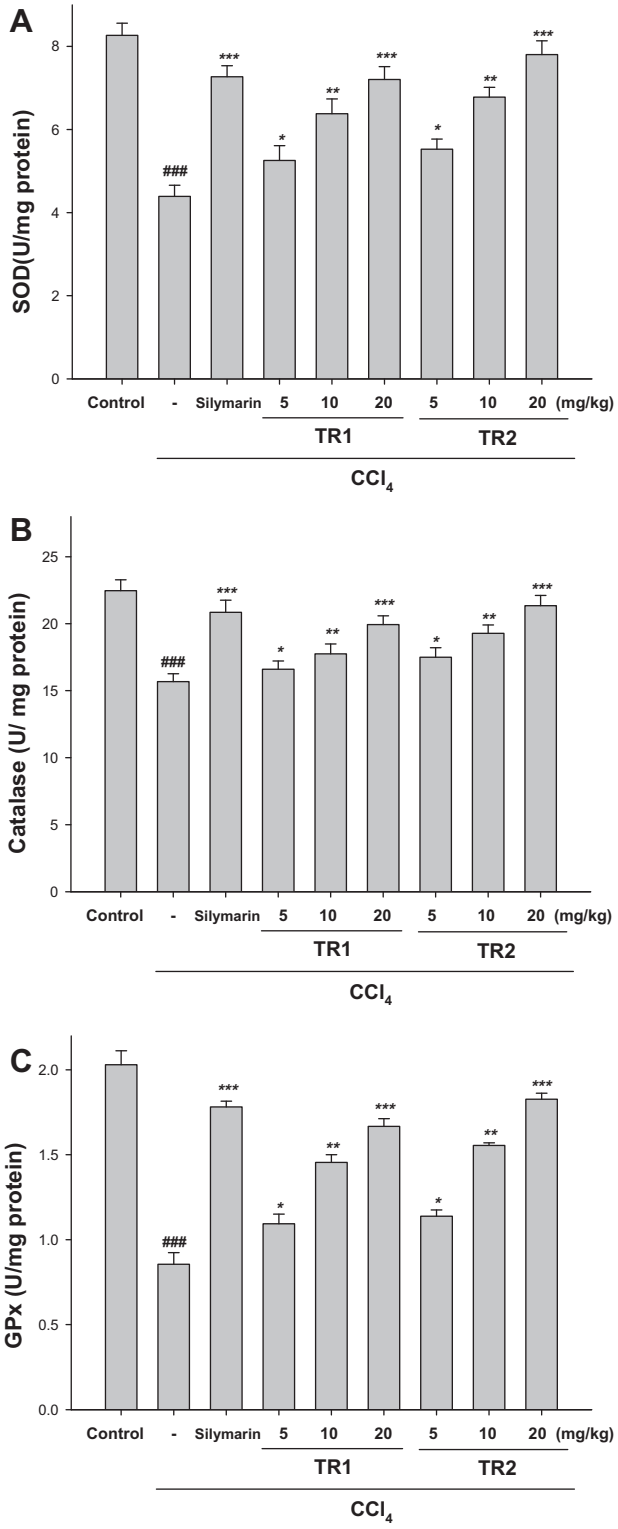

Fig. 3. Effect of **TR1** and **TR2** on the hepatic antioxidant enzymes activities in CCl_4-treated mice. SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase. The values are reported as the mean ± S.E.M. of six mice per group. $^{###}p < 0.01$, compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the CCl_4 group.

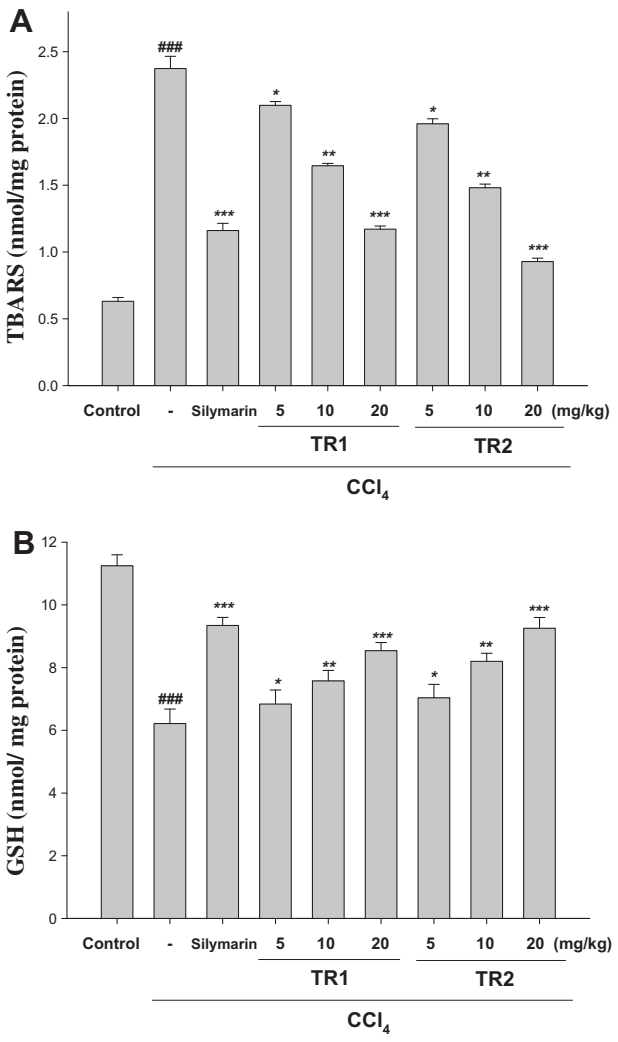

Fig. 4. Effect of **TR1** and **TR2** on hepatic lipid peroxidation (A) and GSH levels (B) in CCl_4-injected mice. The values are reported as the mean ± S.E.M. of six mice per group. $^{###}p < 0.01$, compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the CCl_4 group.

the control group. However, pretreatment of **TR1** and **TR2** reduced the NO production in CCl_4-treated mice. NO production in the control group was 2.79 ± 0.37 μM, while it was 12.45 ± 0.28 μM with CCl_4 treatment. However, the NO production in the CCl_4-treated mice was significantly ($p < 0.001$) decreased to 4.61 ± 0.27 and 3.65 ± 0.22 μM with 20 mg/kg **TR1** and **TR2** pretreatment, respectively. Silymarin treated mice also showed significant ($p < 0.001$) decrease of NO production in serum compared with the CCl_4 group.

3.7. Effect of *TR1* and *TR2* on expressions of iNOS, COX-2, and CYP2E1 in CCl_4-treated mouse liver

We investigated the changes of the activation of iNOS and COX-2 by **TR1** and **TR2** in CCl_4-treated mice (Fig. 6). The results showed that the CCl_4 treatment stimulates activation of iNOS and COX-2 compared to the control. However, the treatment of **TR1** and **TR2** decreased the iNOS and COX-2 expression in CCl_4-induced mice. Namely, iNOS and COX-2 expressions were reduced at 20 mg/kg of **TR1** and **TR2**, respectively, compared to CCl_4 treatment alone.

CYP2E1 is a key enzyme for metabolising xenobiotics such as CCl_4 that produces toxic radicals, causing liver injury. Therefore, the suppression of CYP2E1 could result in decreasing tissue injury. As shown in Fig. 6B, the expression of the enzyme was increased by the treatment with **TR1** and **TR2** as compared to CCl_4 treatment alone. The result indicates that the treatment of **TR1** and **TR2**

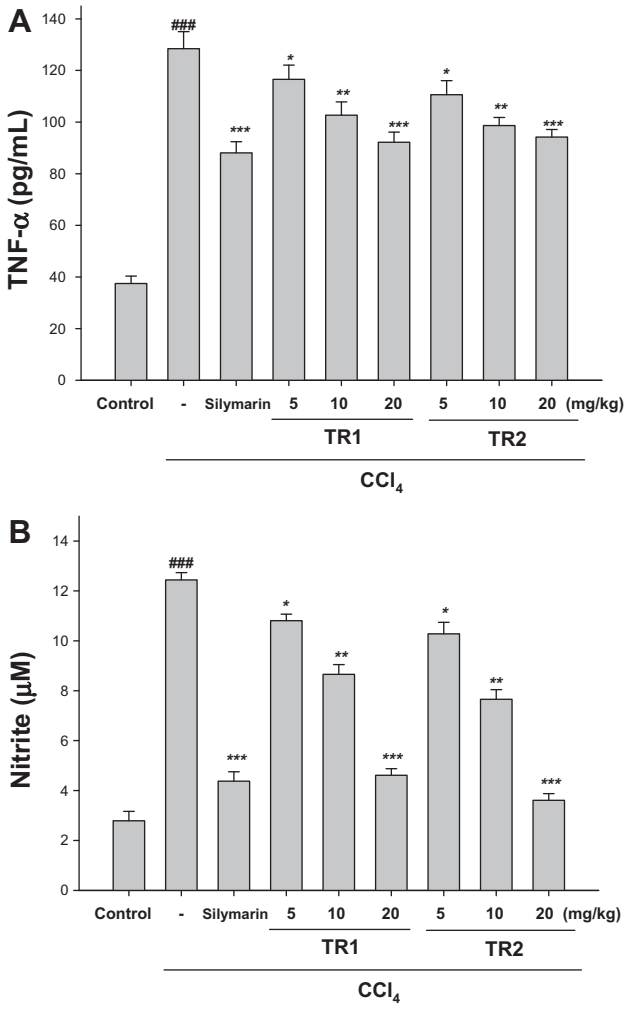

Fig. 5. Effect of **TR1** and **TR2** on the serum TNF-α (A) and NO (B) level in CCl_4-injected mice. The values are reported as the mean ± S.E.M. of six mice per group. $^{\#\#\#}p < 0.01$, compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the CCl_4 group.

(20 mg/kg) increased CYP2E1 expression in CCl_4-induced mice (Fig. 4D).

4. Discussion

CCl_4 is one of the most commonly used hepatotoxins in experimental liver studies. CCl_4 induced liver injuries are the best characterised system of xenobiotic-induced hepatotoxicity and a commonly used model for the screening of hepatoprotective activities of drugs (Huang et al., 2011). The principal cause of CCl_4-induced hepatic damage is lipid peroxidation and decreased activities of antioxidant enzymes and generation of free radicals (Song & Yen, 2003). CCl_4-induced hepatotoxicity is believed to involve two phases. The initial phase involves the metabolism of CCl_4 by cytochrome P450 to trichloromethyl radicals ($CCl_3^{\cdot}$), which leads to membrane lipid peroxidation and finally to cell necrosis. The second phase involves the activation of Kupffer cells, accompanied by the production of proinflammatory mediators. In addition, it has been found that CCl_4-induced toxicity can stimulate endogenous ROS and reactive nitrogen species (RNS), which have been suggested to play important roles in the pathogenesis of hepatotoxicity (Castro et al., 1974). Therefore, we strongly speculate that **TR1** and **TR2** can protect against liver diseases which are caused by ROS. The results of the present study demonstrate that the pre-administration of **TR1** and **TR2** effectively protected mice against CCl_4-induced acute liver damage. The administration of CCl_4 to mice markedly increases serum ALT and AST levels. The increase commonly reflects the severity of liver injury (Dai et al., 2003).

Histopathological examination of the liver sections in CCl_4-injected mice revealed extensive liver injuries, characterised by moderate hepatocellular degeneration and necrosis around the central vein, fatty changes, inflammatory cell infiltration, and the loss of cellular boundaries. However, the histopathological hepatic lesions were markedly ameliorated by the pretreatment with **TR1** or **TR2**, which was in good agreement with the results of serum ALT and AST activity, and hepatic oxidative stress levels. In the present work, substantial increases in serum ALT and AST were observed after the administration of CCl_4; however, the increased levels of enzymes were considerably reduced by pre-treatment with **TR1** and **TR2**, implying that **TR1** and **TR2** tended to prevent damage and suppressed the leakage of enzymes through cellular membranes.

CCl_4 caused a variety of histological changes in the liver, including centrizonal necrosis, portal inflammation, and Kupffer cell hyperplasia. These changes were significantly attenuated by **TR1** and **TR2**. Furthermore, the hepatoprotective effect of **TR1** and **TR2** appeared to be as beneficial as that of silymarin, which is used as a potent hepatoprotective agent. Hence, we suggest that **TR1** and **TR2** have potential clinical application for treating liver diseases.

Hepatoprotective effects may be associated with an antioxidant capacity to scavenge ROS. The balance of intracellular ROS depends on both their production within cells during normal aerobic metabolism and their removal by the antioxidant-defence system that includes nonenzymatic antioxidants (e.g., GSH) and enzymatic antioxidants such as SOD, CAT, and GPx in mammalian cells (Mohammadi & Yazdanparast, 2009). Therefore, the enzymatic antioxidant activities and the inhibition of free-radical generation are important in terms of protecting the liver from CCl_4-induced damage (Huang et al., 2012). Although in the antioxidant paradox, CCl_4 may cause oxidative stress and the consequent upregulation of antioxidant enzymes, rendering cells more resistant to subsequent oxidative damage (Ko, Lee, & Lim, 2006). Our results indicated decreased GSH, SOD, CAT, and GPx levels in mice liver in response to the CCl_4 treatment, while **TR1** and **TR2** pre-treatments maintained normal levels of all three antioxidant enzymes. GSH constitutes the first line of defence against free radicals, and it is a critical determinant of the tissue susceptibility to oxidative damage. It has been reported that GSH plays a key role in detoxifying the reactive toxic metabolites of CCl_4 and that liver necrosis begins when the GSH stores are depleted (Hou, Qin, & Ren, 2010).

The liver is a major inflammatory process contributes to the pathological events after the exposure to various hepatotoxins. Kupffer cells release pro-inflammatory mediators either in response to necrosis or as a direct action by the activated hepatotoxins in CCl_4-induced acute hepatic injury (Mu et al., 2009). TNF-α is rapidly produced by macrophages in response to liver damage. An increase in the TNF-α level has correlated with the histological evidence of hepatic necrosis and an increase in serum aminotransferase levels. TNF-α also stimulates the release of cytokines from macrophages and induces the phagocyte oxidative metabolism and NO production (Ahmed, Mahmoud, Ouf, & El-Fathaah, 2011). The study confirmed a significant increase in serum TNF-α expression after CCl_4 administration. These alterations were attenuated by **TR1** and **TR2** pre-treatment, which suggests that **TR1** and **TR2** suppress TNF-α secretion and/or enhance its degradation.

NO is a highly reactive oxidant that is produced through the action of iNOS and plays a role in a number of physiological processes. INOS overproduction occurs in livers with CCl_4-induced

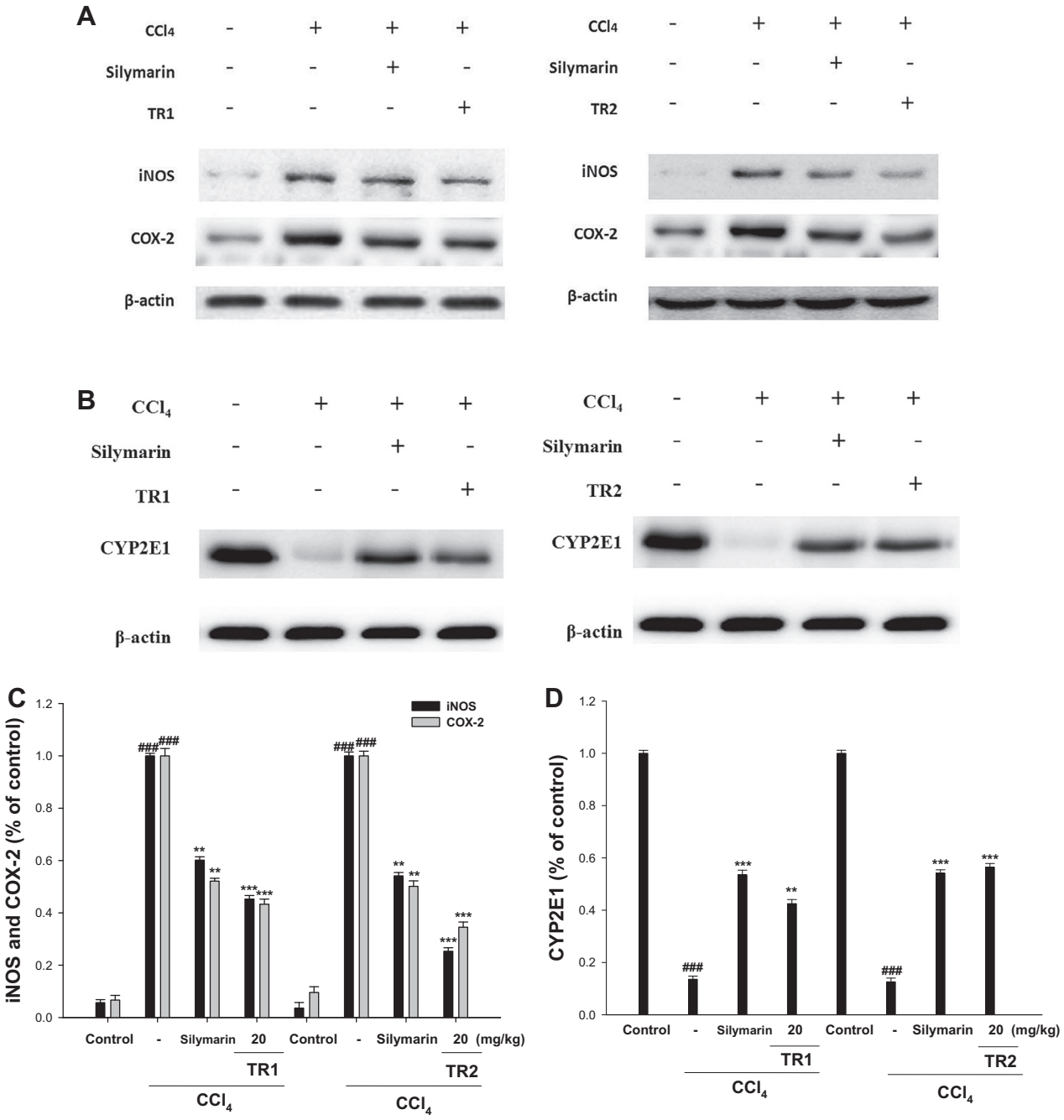

Fig. 6. Effect of **TR1** and **TR2** on CCl_4-injected the expression of iNOS, COX-2 (A), and CYP2E1 (C) in mice. The relative intensities were evaluated by iNOS, COX-2 (B), and CYP2E1 (D) proteins with the use of the Kodak Molecular Imaging Software. β-Actin was used as internal control for equal loading of proteins. The values are reported as the mean ± S.E.M. of six mice per group. $^{###}p < 0.01$, compared with the control group; $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the CCl_4 group.

acute liver injury, and suggests that iNOS acts as a mediator in the pathogenesis of hepatotoxicity in mice (Liong, Xiao, Lau, Nanji, & Tipoe, 2011). However, iNOS expression usually occurs after inflammatory responses. INOS has been implicated as a mediator of cellular injury at sites of inflammation, including liver ischaemia/reperfusion injury. Under this circumstance, NO reacts with superoxide and generates ROS, thereafter modifying bioorganic molecules. ROS leads to extracellular matrix (ECM) degradation and leukocyte migration across ECM proteins (Domitrović & Jakovac, 2010). The study confirmed a significant decrease in the serum TNF-α and NO expressions as a result of **TR1** and **TR2** pre-treatment after CCl_4 administration.

The induction of cyclo-oxygenase by the inflammatory response is a secondary effect of CCl_4-induced hepatotoxicity. COX-2 is thought to be the predominant cyclo-oxygenase involved in inflammatory responses (Choi et al., 2011). In other words, the reduction of excess NO generation as well as inhibition of iNOS and COX-2 protein expression might be associated with the prevention and the treatment of oxidative stress-induced inflammatory diseases. Our data showed an increase in the expression of COX-2 protein after CCl_4 administration. **TR1** and **TR2** attenuated COX-2 expression, suggesting suppression of inflammatory response.

CCl_4 is mainly metabolised to highly reactive trichloromethyl free radicals by CYP2E1. These reactive free radicals induce liver damage by triggering a chain of cellular events. These findings suggest that CYP2E1 suppression could reduce reactive metabolite formation and thus reduce tissue injury. For example, hispidin

analogue davallialactone (CYP2E1 inhibitors) effectively inhibits CCl_4-induced hepatotoxicity in mice (Risal et al., 2012). Our data showed that suppression of CYP2E1 by **TR1** and **TR2** in CCl_4-induced mice was an important aspect of the hepatoprotective effect. **TR1** and **TR2** were significantly beneficial in the prevention of CCl_4-induced liver toxicity, possibly by scavenging reactive free radicals, boosting the endogenous antioxidant system, inhibiting proinflammatory cytokines *via* the downregulation of iNOS, and COX-2, and activating CYP2El expression in CCl_4-induced mice.

The dry matter of fermented filtrate (DMF) from submerged cultures of AC and aqueous extracts from fruiting bodies of AC have been reported to possess hepatoprotective activity against liver diseases induced by CCl_4 (Song & Yen, 2003). DMF from submerged cultures of AC and aqueous extracts from fruiting bodies of AC could reduce antioxidant enzymes and the GSH/GSSG ratio was significantly improved by the oral pretreatment of rats. In addition, the fruiting bodies and mycelia of AC possessed protective activity against liver hepatitis and fatty liver induced by acute hepatotoxicity of alcohol (Dai et al., 2003). The treatment with AC notably prevented the ethanol-induced elevation of levels of serum AST, ALT, alkaline phosphatase, and bilirubin to an extent that was comparable to silymarin (Ko et al., 2006). Silymarin also prevents CCl_4-induced lipid peroxidation and hepatotoxicity by decreasing the metabolic activation of CCl_4 and by acting as a chain-breaking antioxidant in mice (Lettéron et al., 1990), which the mechanism of silymarin was similarity as **TR1** and **TR2**. In our previous paper, antrosterol from AC whole submerged had hepatoprotective activity against liver diseases induced by CCl_4 (Huang et al., 2011).

In summary, **TR1** and **TR2** from AC protect the liver from CCl_4-induced oxidative stress and tissue injuries, which might be due to the antioxidant properties and the inhibition of the inflammatory response and lipid peroxidation. The possible role of AC as a promising therapeutic in human oxidative stress and inflammatory liver disease deserves consideration. The potential of using **TR1** and **TR2** in both experimental designs and practical applications should be examined in the near future.

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