Protective effect of antrosterol from *Antrodia camphorata* submerged whole broth against carbon tetrachloride-induced acute liver injury in mice

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ABSTRACT

The hepatoprotective potential of antrosterol (ergostatrien-3β-ol, ST1) from *Antrodia camphorata* (AC) against carbon tetrachloride (CCl_4)-induced liver damage was evaluated in preventive models in mice. Pretreatment with ST1 markedly 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)] were significantly increased after treatment with CCl_4 *in vivo*. In addition, ST1 decreased the level of nitric oxide (NO) production and tumour necrosis factor-alpha (TNF-α) in CCl_4-treated mice. In this study, these results pointed out that ST1 can inhibit lipid peroxidation, enhance the activities of antioxidant enzymes, decreases the TNF-α level, nitric oxide production and inducible nitric oxide synthase (iNOS), and cyclo-oxygenase-2 (COX-2) expressions. Therefore, it was speculated that ST1 protects mice from liver damage through their anti-inflammation capacity.

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1. Introduction

The reactive oxygen species (ROS) play an important role in the degenerative or pathological processes of various diseases, such as ageing, cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts, and inflammation. The production of ROS can be induced by a variety of factors such as ionizing radiation, exposure to drug and xenobiotics or enhanced by other factors (Bruccoleri et al., 1997). CCl_4, an extensively used xenobiotic, is thought to produce a highly reactive trichloromethyl radical by cytochrome P_{450} system in liver, consequently causes lipid peroxidation that leads to hepatocellular membrane damage and followed by the release of inflammatory mediators from activated hepatic macrophages, which are thought to potentiate CCl_4-induced hepatic damage (Kuo et al., 2010). These activated macrophages released inflammatory mediators including TNF-α and NO have been implicated in liver damage induced by a number of different toxicants (Celik, Temur, & Isik, 2009).

Antrodia camphorata (AC), also called *A. cinnamomea*, is composed of fruiting bodies, mycelium and spores. The fruiting body of AC is well known in Taiwan as a traditional Chinese medicine. It has been used for the treatment of food and drug intoxication, diarrhoea, abdominal pain, hypertension, skin itching, and cancer. The fruiting body and cultured mycelia of AC contain fatty acids, lignins, phenylderivatives, sesquiterpenes, steroids, and triterpenoids (Geethangili & Tzeng, 2009). The fermented culture broth had cytotoxic activity against several tumour cell lines (Yeh et al., 2009), anti-inflammation (Huang et al., 2010), vasorelaxation (Wang et al., 2003) and anti-hepatitis B virus activity (Lee et al., 2002). The filtrate in submerged culture also had protective effects against CCl_4-induced hepatic toxicity and high antioxidant properties (Song & Yen, 2003). However, little information is available on the protective effects against CCl_4-induced hepatic toxicity effects of antrosterol (ST1) (Fig. 1A). To our knowledge, this study is the first report that demonstrates the anti-inflammatory effects of ST1 in liver protection and regulation of iNOS and COX-2 in CCl_4-induced liver injury model by AC component.
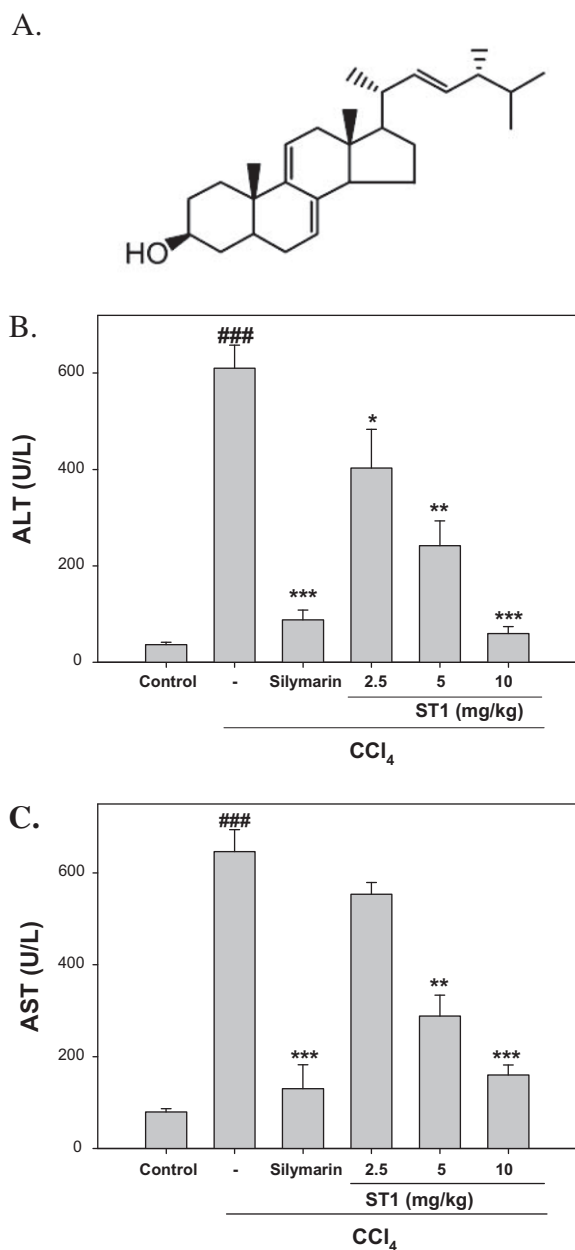
2. Materials and methods

2.1. Chemicals

Carbon tetrachloride was purchased from Merck (Darmstadt, Germany). Silymarin, malondialdehyde, and other chemicals were

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A.

B.

C.

Fig. 1. Chemical structure of ST1 (A) and the effect of ST1 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 $^{**}p < 001$ and $^{***}p < 0.001$ compared with the CCl$_4$ group.

purchased from Sigma Chemical Co (Steinheim, Germany). Biochemical assay kits for measurement of ALT and AST contents were purchased from Randox Laboratories (Crumlin, United Kingdom). TNF-α was purchased from Biosource International Inc., (Camarillo, CA, USA).

2.2. Fungus material

Freeze-dried powder of AC of the submerged whole broth (Batch No. MZ-247) was provided by the Biotechnology Center of Grape King Inc., Chung-Li City, Taiwan, Republic of China.

2.3. Isolation and determination of the active compound

Freeze-dried powder of AC of the submerged whole broth (1.6 kg) was extracted three times with methanol (16 l) at room temperature (1 day each). The methanol extract was evaporated *in vacuo* to give a brown residue, which was suspended in H$_2$O (1 l), and then partitioned (3 times) with 1 l of ethyl acetate. The EtOAc fraction (95 g) was chromatographed on silica gel using mixtures of hexane and EtOAc of increasing polarity as eluents and further purified with HPLC. ST1 (5.4 g) was eluted with 10% EtOAc in hexane, and recrystallization with EtOH (Shao et al., 2008).

2.4. Animal treatment

Six to eight week old males imprinting control region (ICR) mice were obtained from the BioLASCO Taiwan Co., Ltd. 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 and negative control groups were administered with distilled water. The positive control group was administered with silymarin (25 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 ST1 (2.5, 5, and 10 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 anesthetized with ethyl ether and blood samples were collected through their carotid arteries. The mortality rate and body weight were recorded daily.

2.5. Assessment of liver functions

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

2.6. Histopathological examination

Small pieces of liver, fixed in 10% buffered formalin were processed for embedment in paraffin. Sections of 4–5 μm were cut and stained with heamatoxylin 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 analyzed to check the hepatoprotective activity of ST1 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$_2$O$_2$ 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. Activity was evaluated from a calibration curve, and the enzyme activity was defined as nanomoles of NADPH oxidized 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 (Ellman, 1959). Briefly, 720 μl of the liver homogenate in 200 mM Tris–HCl buffer (pH 7.2) was 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 10,000g for 5 min at 4 °C. Supernatant (330 μl) was taken in a tube and 660 μl of Ellman's reagent (DTNB) solution was added to it. Finally the absorbance was taken at 405 nm.

2.9. Lipid peroxidation intermediates

Thiobarbituric acid reactive substances (TBARS), in particular malondialdehyde (MDA), are 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, according to as previously reported (Tatum & Chow, 1996). Briefly, 0.4 ml of the treated cell or liver extract was 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 at 90 °C water for 45 min, cooled, added the equal volume of n-butanol, centrifuged and then the absorbance of the supernatant 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. Total protein assay

Protein content in each sample was determined by a bicinchoninic acid (BCA) protein assay kit (Pierce).

2.11. 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.

2.12. 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 (Recknagel, Glende, & Britton, 1991). Serum samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulphate (300 g/l) to a final concentration of 15 g/l. After centrifugation at 10,000g for 5 min at room temperature, 100 μl supernatant was applied to a microtiter plate well, followed by 100 μl of Griess reagent (1% sulphanilamide 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, Orleans Drive, Sunnyvale, CA).

By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured by absorbance at 540 nm.

2.13. Western blot analysis

Liver tissues were homogenized 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 microgrammes of protein was fractionated on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were incubated with primary antibodies overnight at 4 °C using 1:1000 dilution of goat polyclonal anti-rabbit iNOS, COX-2 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., Buckinghamshire, 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.

2.14. Statistical analysis

All data were presented as mean ± standard deviation (SD) from three independent experiments. Means of triplicates were calculated. Student's t test was used for comparison between two treatments. A difference was considered to be statistically significant when $p < 0.05$, $p < 0.01$ or $p < 0.001$.

3. Results

### 3.1. Effect of ST1 on hepatotoxicity in CCl$_4$-treated mice

Several hepatic enzymes in serum such as AST and ALT were used as the biochemical markers for the 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 ST1 at three different doses (2.5, 5 and 10 mg/kg) significantly prevented the increased serum levels of ALT and AST. Silymarin (a positive control) at a dose of 25 mg/kg also prevented the elevation of ALT and AST. In this study, mice treated with CCl$_4$ developed significant hepatic damage as manifested by a significant increase in activities of AST and ALT that are indicators of hepatocyte damage and loss of functional integrity.

3.2. Histopathology of the liver

Fig. 2 showed that that CCl$_4$ could induce histological changes including increased degeneration, necrosis, hepatitis and portal tri-aditis. All mice except those in the control group exhibited the ballooning degeneration in the centrolobular zone and the necrosis of hepatocytes (Fig. 2). The CCl$_4$-induced damage suffered more severely than other groups pretreated with ST1. It seems likely that CCl$_4$ administration cause oxidative stress in liver via the generation of free radicals whereas ST1 ameliorates the liver injuries by scavenging of free radicals, which is further confirmed by the reduced amount of histopathological injury.

3.3. Effect of ST1 on antioxidant enzymes activities in CCl$_4$-treated mice liver

The hepatic antioxidant enzyme activities (SOD, CAT and GPx) are shown in Fig. 3. The activities of SOD, CAT and GPx were
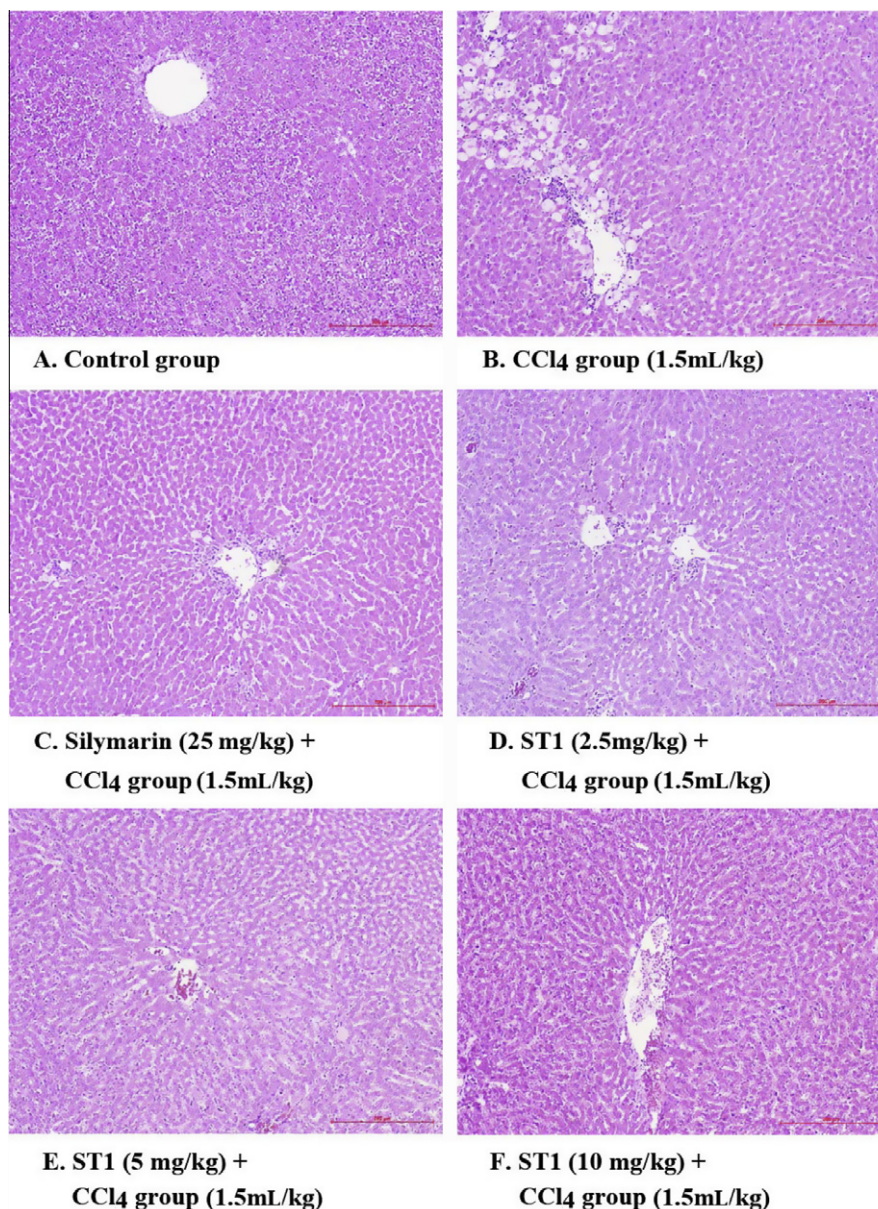
Fig. 2. Effect of ST1 on the CCl₄-induced liver damage. Sections were stained with heamatoxylin-eosin (100×) and observed under the light microscopy. (A) control; (B) received CCl₄ (1.5 ml/kg); (C) silymarin (25 mg/kg) + CCl₄ (1.5 ml/kg); (D) ST1 (2.5 mg/kg) + CCl₄ (1.5 ml/kg); (E) ST1 (5 mg/kg) + CCl₄ (1.5 ml/kg); (F) ST1 (10 mg/kg) + CCl₄ (1.5 ml/kg).

significant decreased in CCl₄-treated mice, comparing to the control. Mice pretreated with ST1 at 2.5, 5 and 10 mg/kg showed significant increase in SOD when compared to CCl₄ group. Mice pretreated with ST1 at 10 mg/kg showed significant increase in CAT when compared to CCl₄ group. Mice pretreated with ST1 at 10 mg/kg showed significant ($p < 0.001$) increase in GPx when compared to CCl₄ group. Silymarin-treated mice (The group of reference protective drug) also showed significant increase in SOD, CAT, and GPx when compared to CCl₄ treated mice.

3.4. Effect of ST1 on lipid peroxidation in CCl₄-treated rat liver

As shown in Fig. 4A, hepatic levels of TBARS were assessed as an indicator of lipid peroxidation in the tissue. CCl₄ treatment significantly increased the level of TBARS in the liver. CCl₄ alone treated mice was observed a significant increase ($p < 0.001$) in tissue

TBARS level. CCl₄-induced elevation of tissue TBARS concentration was lowered significantly by the i.p. pre-treatment of the mice with ST1. ST1 at 2.5, 5 and 10 mg/kg significantly prevented the increase in TBARS level when compared to CCl₄ group; Silymarin also protected the liver from elevating TBARS levels and kept TBARS levels in normal values.

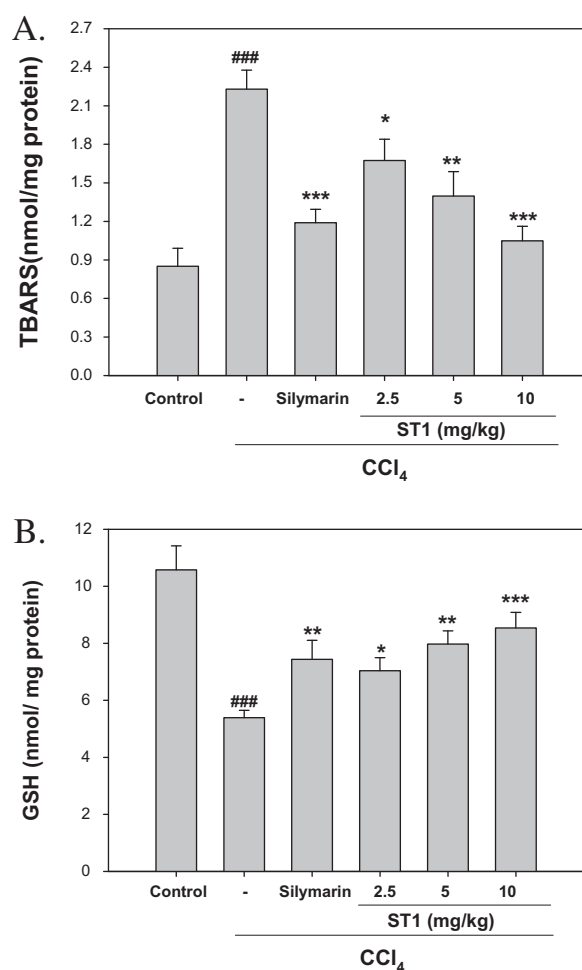### 3.5. Effect of ST1 on cellular GSH levels in CCl₄-treated mice

The CCl₄-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 ST1 at the dose of 2.5, 5 and 10 mg/kg resulted in significant increase of GSH content when compared to CCl₄ treated mice. Silymarin (25 mg/kg) treated mice also showed significant ($p < 0.001$) increase in GSH level in liver compared with CCl₄ group.

A.

B.

C.
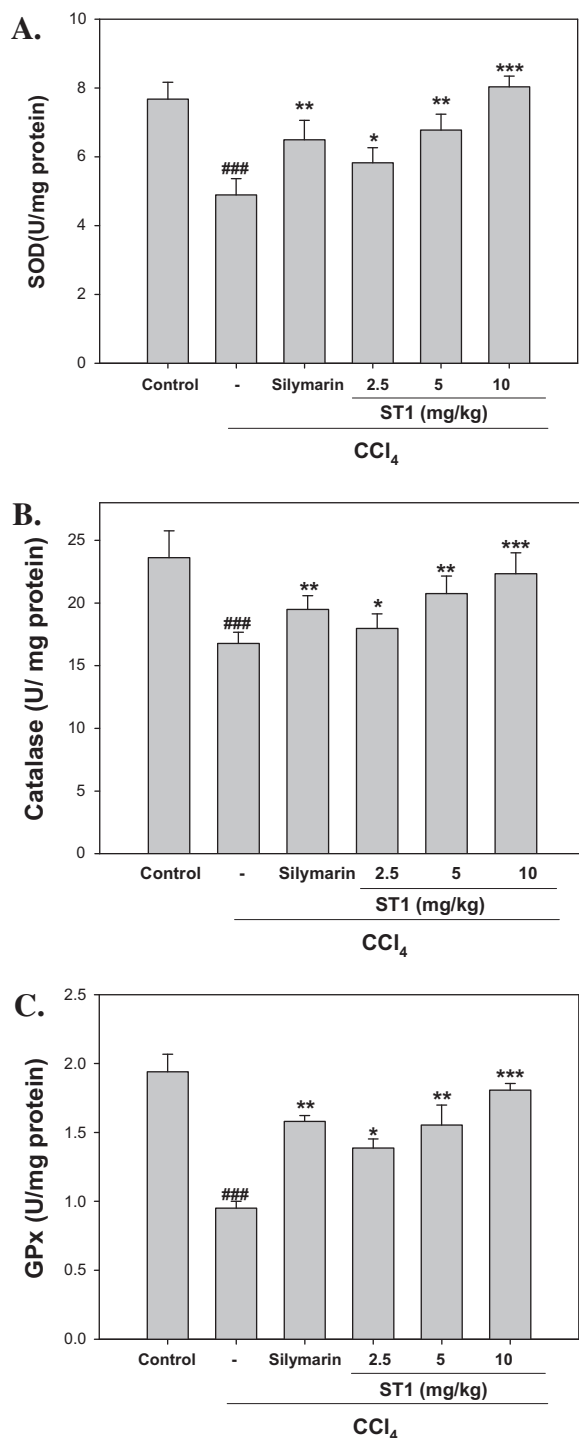
Fig. 3. Effect of ST1 on the hepatic antioxidant enzymes activities in CCl$_4$-treated mice. SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase. The values are reported as the mean ± S.E.M. of six mice per group. $^{###}p < 0.01$, compared with the control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the CCl$_4$ group.

A.

B.

Fig. 4. Effect of ST1 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. $^{###}p < 0.05$, compared with the control group; $^{**}p < 0.01$ compared with the CCl$_4$ group.

3.6. Effect of ST1 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 87.45 ± 4.88 pg/ml in the control group. The CCl$_4$-treatment caused significant ($p < 0.01$) increase in the level of TNF-α in the serum when compared with control group. The pretreatment of ST1 at the dose of 2.5, 5 and 10 mg/kg resulted in significant decrease of

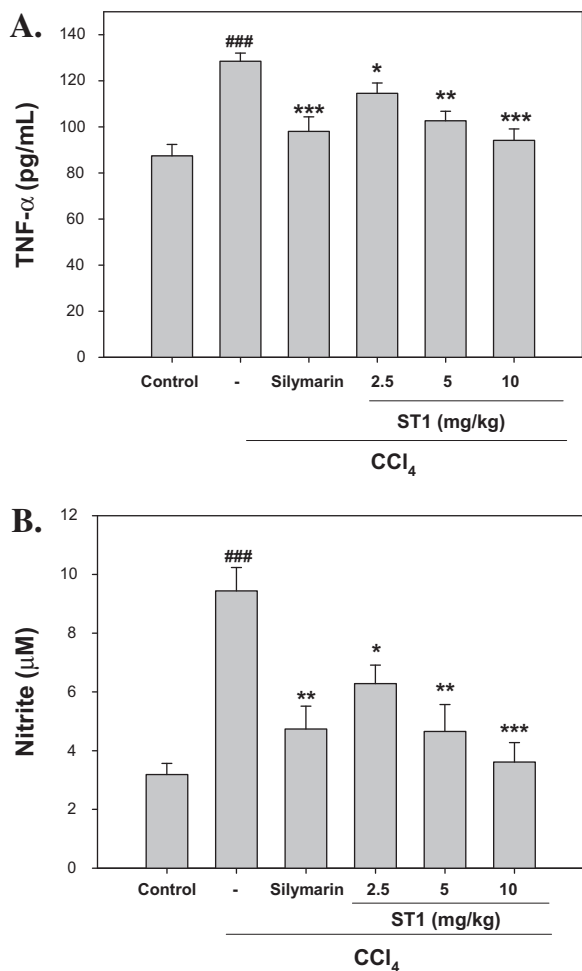TNF-α level when compared to CCl$_4$-treated mice. Silymarin (25 mg/kg) treated mice also showed significant ($p < 0.05$) decrease in TNF-α level in serum compared with CCl$_4$-treated mice. As shown in Fig. 5B, the production of NO in mice serum was significantly increased in CCl$_4$-treated mice comparing to the control group. However, pretreatment of ST1 reduced the NO production in CCl$_4$-treated mice. For example, NO production in the control group was 3.18 ± 0.34 μM, while it was 9.44 ± 0.79 μM with CCl$_4$ treatment. However, the NO production in the CCl$_4$-treated mice was significantly ($p < 0.001$) decreased from 6.28 ± 0.63, 4.66 ± 0.91 to 3.61 ± 0.66 μM with 2.5, 5, and 10 mg/kg ST1 pretreatment, respectively. Silymarin (25 mg/kg) treated mice also showed significant ($p < 0.05$) decrease of NO production in serum compared with CCl$_4$ group.

3.7. Effect of ST1 on activities of iNOS and COX-2 in CCl$_4$-treated mice liver

The changes of the activation of iNOS and COX-2 by ST1 in CCl$_4$-treated mice (Fig. 6) were investigated. The relative intensities of bands obtained from Western blot were calculated with the use of the Kodak Molecular Imaging Software (Tokyo, Japan). The results showed that CCl$_4$ treatment stimulates to increase activation of iNOS and COX-2. For example, in CCl$_4$ treatment group, the relative intensity of iNOS and COX-2 band was increased by
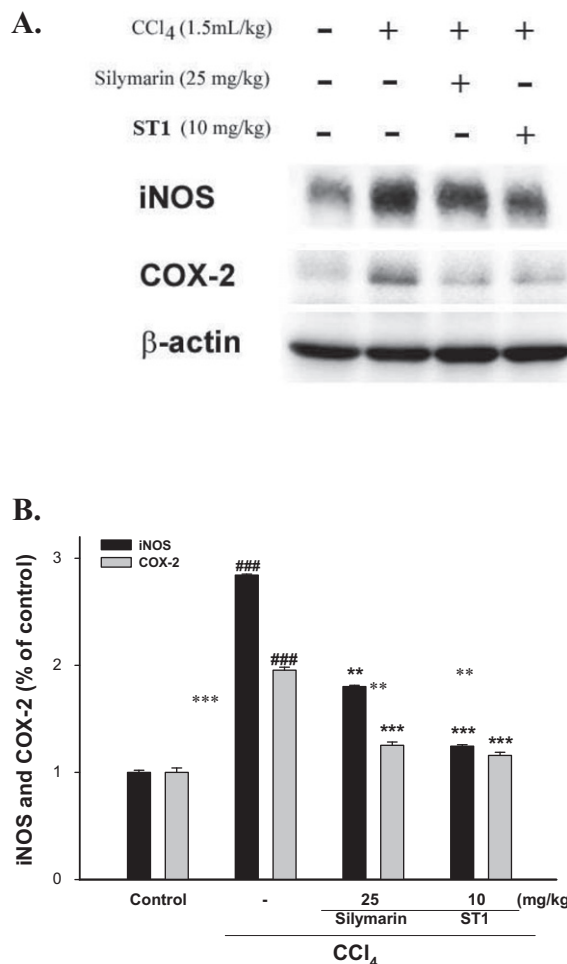
Fig. 5. Effect of ST1 on the serum TNF-α (A) and NO (B) level in CCl₄-injected mice. The values are reported as the mean ± S.E.M. of six mice per group. $^{###}p < 0.05$ as compared to control group; $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to CCl₄-treated group.

Fig. 6. Effect of ST1 on CCl₄-injected the activation of iNOS and COX-2 (A) in mice. The relative intensities were evaluated by iNOS and COX-2 (B) proteins with the use of the Kodak Molecular Imaging Software. The values are reported as the mean ± S.E.M. of six mice per group. $^{###}p < 0.05$ as compared to control group; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to CCl₄ group.

2.84- and 1.95-fold, compared to the control. However, the treatment of ST1 decreased the iNOS and COX-2 expression in CCl₄-induced mice. Namely, the relative intensities of bands about iNOS and COX-2 expressions were reduced by 1.24- and 1.16-fold at 10 mg/kg of ST1, respectively, compared to CCl₄ treatment alone.

4. Discussion

In this manuscript, it was strongly speculated that ST1 can protect against diseases which are caused by ROS, because it has a radical scavenging activity. The results of the present study demonstrate that the pre-administration of ST1 effectively protected mice against CCl₄-induced acute liver damage. Administration of CCl₄ to mice markedly increases serum ALT and AST levels. This increase commonly reflects the severity of liver injury (Lin, Yao, Lin, & Lin, 1996). The leakage of large quantities of enzymes into the blood stream was associated with massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver. In the present work, substantial increases in serum ALT and AST were observed after administration of CCl₄, however, the increased levels of enzymes were considerably reduced by pre-treatment with ST1, implying that ST1 tended to prevent damage and suppressed the leakage of enzymes through cellular membranes. 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 CCl₄ (Song & Yen, 2003). Both of them could reduce GSH-dependent enzymes (GPx, GSH reductase and GSH-S-transferase), and the GSH/GSSG ratio was significantly improved by the oral pre-treatment of rats with DMF. Also, scientific research showed that both 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). Using acute ethanol-intoxicated rats as an experimental model, the hepatoprotective effects of AC, a traditional Chinese fungi drug for liver diseases, on liver injury induced by ethanol (Liu et al., 2007) were compared. Treatment with AC notably prevented the ethanol-induced elevation of levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and bilirubin to an extent that was comparable to the standard drug silymarin.

Many studies have shown that the hepatoprotective effects may be associated with an antioxidant capacity to scavenge reactive oxygen species (Sabir & Rocha, 2008). Antioxidant enzymes (SOD, CAT and GPx) offer protection against oxidative tissue damage. Although in the antioxidant paradox, CCl₄ may cause oxidative stress and the consequent up-regulation of antioxidant enzymes, to rendering cells more resistant to subsequent oxidative damage (Halliwell & Gutteridge, 1990), however, this phenomenon was not observed. Our results indicated decreased SOD, CAT, and GPx

levels in mice liver in response to CCl_4 treatment, while ST1 pre-treatments kept levels of all three antioxidant enzymes (Fig. 3) to the normal ones. This may be due to the inhibitory effects on cytochrome P_{450} and/or promotion of its glucuronidation, both related to the early stage in CCl_4-induced liver injury. GSH constitutes the first line of defense against free radicals and 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 (Williams & Burk, 1990). In this study, CCl_4 treatment decrease the hepatic GSH levels and ST1 pre-treatments also restored the hepatic GSH levels to the normal ones (Fig. 4). The effect could be due either to the *de novo* synthesis of GSH, its regeneration, or both.

The liver is a major inflammatory organ, and inflammatory processes contribute to a number of pathological events after exposure to various hepatotoxins. Kupffer cells release pro-inflammatory mediators either in response to necrosis or as a direct action by the activated hepatotoxins, which are believed to aggravate CCl_4-induced hepatic injury (Badger, Sauer, Hoglen, Jolley, and Sipes, 1996). TNF-α, a pleiotropic pro-inflammatory cytokine, is rapidly produced by macrophages in response to tissue damage. While low levels of TNF-α may play a role in cell protection, excessive amounts cause cell impairment. An increase in the TNF-α level has been directly correlated with the histological evidence of hepatic necrosis and the increase in the serum aminotransferase levels (Bruccoleri et al., 1997). DeCicco, Rikans, Tutor, and Hornbrook (1998) have reported the stimulation of TNF-α production in both serum and liver following CCl_4 administration, and it is suggested that $CCl_3\cdot$ activates Kupffer cells to release TNF-α. TNF-α also stimulates the release of cytokines from macrophages and induces the phagocyte oxidative metabolism and nitric oxide production (DeCicco et al., 1998). Nitric oxide is a highly reactive oxidant that is produced through the action of iNOS, and plays a role in a number of physiological processes, such as, vasodilation, neurotransmission, and nonspecific host defense (Yen, Lai, & Chou, 2001). Nitric oxide can also exacerbate oxidative stress by reacting with reactive oxygen species, particularly with the superoxide anion and forming peroxynitrite. This study confirmed a significant increase in the serum TNF-α protein expression after CCl_4 administration. These alterations were attenuated by ST1 pre-treatment (Fig. 5), which suggests that ST1 suppresses TNF-α protein secretion and/or enhances its degradation.

ST1 blocked the reduction of serum NO level in CCl_4-treated mice. There are two possible explanations for the observed decrease in NO levels after CCl_4 treatment in our study. First, gene expression of nitric oxide synthase (NOS) was reduced. Second, the NOS system (enzyme protein, substrates, or cofactors) was damaged, thus decreasing the NO production. Third, NO usage increased after CCl_4 treatment. It is possible that another mechanism of protective action of ST1 against CCl_4^- induced hepatotoxicity is due to the increased NO production. Several studies have found that NO protected against CCl_4-induced liver injury using a NOS knockout mice or a NOS inhibitor (Wink et al., 1996). The mechanism underlying the protective effects of NO in CCl_4-induced hepatotoxicity has not been elucidated and may be related to its antioxidant properties. NO has also been shown to interfere directly with the progression of lipid peroxidation, which may contribute to its protective actions in the present work. NO is a short-lived signalling molecule capable of regulating many physiological and pathological processes. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed; iNOS is triggered in many cell types by cytokines such as TNF-α or interferon-γ. Endogenous NO, produced by an early and transient activation of constitutive NOS, protects both hepatocytes and endothelial cells against reperfusion injury in the liver (Liu et al., 2007). 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 reactive nitrogen species (ROS), thereafter modifying bioorganic molecules. ROS leads to extracellular matrix (ECM) degradation and the leucocyte migration across ECM proteins.

In conclusion, ST1 protects 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. It's possible role as a promising therapeutic in human oxidative stress and inflammatory liver disease deserves consideration. The potential for using ST1 in experimental designs and practical applications should be examined further.

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