Running Head: The hepatoprotective effect of antrosterol in mice

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

Guan-Jhong Huang[a], Jeng-Shyan Deng[b], Shyh-Shyun Huang[a], Yi-Yuan Shao[c], Chin-Chu Chen[c], Yueh-Hsiung Kuo[*, d]

[a]Institute of Chinese Pharmaceutical Sciences, China Medical University, Taichung 404, Taiwan

[b]Department of Health and Nutrition Biotechnology, Asia University, Taichung 413, Taiwan

[c]Department of Food Science, Nutrition, and Nutraceutical Biotechnology, Shih Chien University, Taipei, Taiwan

[d]Tsuzuki Institute for Traditional Medicine, China Medical University, Taichung, Taiwan

[*]Corresponding author

Dr. Kuo Yueh-Hsiung

Tel.: +886 4 2207-1693; Fax: +886 4 2207-1693

E-mail address: yhkuo@ntu.edu.tw

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. We evaluated 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 tumor 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 cyclooxygenase-2 (COX-2) expressions. Therefore, we speculate that **ST1** protects mice from liver damage through their anti-inflammation capacity.

KEY WORDS: Chinese herb; Antrosterol; anti-inflammation; MDA; NO; TNF-*α*.

1. Introduction

The reactive oxygen species (ROS) play an important role in the degenerative or pathological processes of various diseases, such as aging, 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, 2007).

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, diarrhea, abdominal pain, hypertension, skin itching, and cancer. The fruiting body and cultured mycelia of AC contain fatty acids, lignans, 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. 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, 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_2O (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.

6-8 weeks male 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℃, 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 1700×*g* (Beckman GS-6R, Germany) at 4℃ 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℃ 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 hematoxylin 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 and 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 (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 and 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 μLwith 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℃. 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℃ 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 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 was applied to a microtiter 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 color 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℃. Fifty micrograms 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℃ 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, U.K.). 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 1C). 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 triaditis. 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 significant decreased in CCl_4-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 CCl_4 group. Mice pretreated with **ST1** at 10 mg/kg showed significant increase in CAT when compared to CCl_4 group. Mice pretreated with **ST1** at 10 mg/kg showed significant ($p < 0.001$) increase in GPx when compared to CCl_4 group. 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 ST1 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 treatment significantly increased the level of TBARS in the liver. CCl_4 alone treated mice was observed a significant increase ($p < 0.001$) in tissue TBARS level. CCl_4-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 CCl_4 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 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 **ST1** at the dose of 2.5, 5 and 10 mg/kg resulted in significant increase of GSH content when compared to CCl_4 treated mice. Silymarin (25 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 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.

We investigated the changes of the activation of iNOS and COX-2 by **ST1** in CCl_4-treated mice (Fig. 6). 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 2.84- and 1.95-fold, compared to the control. However, the treatment of **ST1** decreased the iNOS and COX-2 expression in CCl_4-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 CCl_4 treatment alone.

4. Discussion

In this manuscript, we 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

CCl_4-induced acute liver damage. Administration of CCl_4 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 CCl_4, 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 CCl_4 (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 pretreatment 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, we compared the hepatoprotective effects of AC, a traditional Chinese fungi drug for liver diseases, on liver injury induced by ethanol (Liu et al., 2007). 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 (Sabis & Rocha, 2008). Antioxidant enzymes (SOD, CAT and GPx) offer protection against oxidative tissue damage. Although in the antioxidant paradox, CCl_4 may cause oxidative stress and the consequent up-regulation of antioxidant enzymes, to rendering cells more resistant to subsequent oxidative damage (Halliwell & Gutteridge, 1990), we did not observed this phenomenon. 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, et al., 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 *et al.* (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 signaling 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 ischemia/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 leukocyte 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.

Acknowledgements:

The authors want to thank the financial supports from the National Science Council (NSC100-2313-B-039-004, NSC100-2815-C-039-026-B, and NSC100-2815-C-039-027-B), China Medical University (CMU) (CMU100-SR-24 and CMU100-SR-25), Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004), and Department of Cancer Research Center of Excellence (DOH100-TD-C-111-005)

References

Aebi, H. (1984). Catalase *in vitro*. *Methods in Enzymology, 105*, 121-126.

Badger, D.A., Sauer, J.M., Hoglen, N.C., Jolley, C.S., & Sipes, I.G. (1996).The role of inflammatory cells and cytochrome P450 in the potentiation of CCl_4-induced liver injury by a single dose of retinol. *Toxicology and Applied Pharmacology, 141*, 507–519.

Bruccoleri, A., Gallucci, R., Germolec, D.R., Blackshear, P., Simeonova, P.,Thurman, R.G., & Luster, M.I. (1997). Induction of early-immediate genes by tumor necrosis factor alpha contribute to liver repair following chemical-induced hepatotoxicity. *Hepatology*, *25*, 133-141.

Celik, I., Temur, A., & Isik, I. (2009). Hepatoprotective role and antioxidant capacity of pomegranate (*Punica granatum*) flowers infusion against trichloroacetic acid-exposed in rats. *Food and Chemical Toxicology*, *47*, 145-149.

Dai, Y.Y., Chuang, C.H., Tsai, C.C., Sio, H.M., Huang, S.C., Chen, J.C., & Hu, M.L. (2003).The protection of *Antrodia camphorata* against acute hepatotoxicity of alcohol in rats. *Journal of Food and Drug Analysis*, *11*, 177–185.

DeCicco, L.A., Rikans, L.E., Tutor, C.G., & Hornbrook, K.R. (1998). Serum and liver concentrations of tumor necrosis factor alpha and interleukin-1beta following administration of carbon tetrachloride to male rats. *Toxicology Letters, 98,* 115-121.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics, 82,* 70–77.

Flohe, L., & Otting, F. (1984). Superoxide dismutase assays. *Methods in Enzymology, 105*, 93 –104.

Geethangili, M., & Tzeng, Y. M. (2009). Review of Pharmacological Effects of *Antrodia camphorata* and its Bioactive Compounds. *Evidence-based Complementary and Alternative Medicine*, doi:10.1093/ecam/nep108.

Halliwell, B., & Gutteridge, J.M.S. (1990). Role of free radical and catalytic metal ions in human disease: an overview. *Methods in Enzymology, 186*, 59-85.

Huang, G.J., Huang, S.S., Lin, S.S., Shao, Y.Y., Chen, C.C., Hou, W.C., & Kuo, Y.H. (2010a). Analgesic effects and the mechanisms of anti-inflammation of ergostatrien-3beta-ol from Antrodia camphorata submerged whole broth in mice. *Journal of Agricultural and Food Chem*istry, *58*, 7445-7452.

Kuo, D. H., Kang, W. H., Shieh, P. C., Chen, F. A., Chang, C.D., Tsai, M. L., Cheng, A.C., Ho, C.T., & Pan, M.H. (2010). Protective effect of *Pracparatum mungo* extract on carbon tetrachloride-induced hepatotoxicity in rats. *Food Chem*istry, *123*, 1007-1012.

Lee, I.H., Huang, R.L., Chen, C.T., Chen, H.C., Hsu, W.C., & Lu, M.K. (2002). *Antrodia camphorata* polysaccharides exhibit anti-hepatitis B virus effects. *FEMS Microbiology Letters, 209*, 63-67.

Lin, S.S., Yao, C.J. Lin, C.C. & Lin, Y.H. (1996). Hepatoprotective activity of Taiwan folk medicine: *Eclipta prostat*e *Linn.* Against various hepatotoxins induced acute hepatoxicity. *Phytotherapy Research, 10,* 483-490.

Liu, D.Z., Liang, Y.C., Lin, S.Y., Lin, Y.S., Wu, W.C., Hou, W.C., & Su, C.H. (2007). Antihypertensive activities of a solid-state culture of *Taiwanofungus camphoratus* (Chang-Chih) in spontaneously hypertensive rats, *Bioscience Biotechnology and Biochemistry 71*, 23–30.

Paglia, E.D., & Valentine, W.N. (1967). Studies on the quantitative and qualitative characterization of erythrocytes glutathione peroxidase. *Journal of Laboratory Clinical Medicine, 70*, 158–169.

Sabir, S.M. & Rocha, J.B.T. (2008).Water-extractable phytochemicals from *Phyllanthus niruri* exhibit distinct *in vitro* antioxidant and *in vivo* hepatoprotective activity against paracetamol-induced liver damage in mice. *Food Chemistry, 111*, 845-851.

Shao, Y.Y., Chen, C.C., Wang, H.Y., Chiu, H.L., Hseu, T.H., & Kuo, Y.H. (2008). Chemical constituents of *Antrodia camphorata* submerged whole broth. *Natural Product Research, 22*, 1151-1157.

Song, T.Y., & Yen, G.C. (2003). Protective effects of fermented filtrate from *Antrodia camphorata* in submerged culture against CCl_4-induced hepatic toxicity in rats. *Journal of Agricultural and Food Chem*istry, *51*, 1571-1577.

Tatum, V. L., & Chow, C. K. (1996). Antioxidant status and susceptibility of sickle erythrocytes to oxidative and osmotic stress. *Free Radical Research, 25*, 133-139.

Recknagel, R. O., Glende, E. A. Jr., & Britton, R. S. (1991). Free radical damage and lipid peroxidation. In: R.G. Meeks, Editor, *Hepatotoxicology* CRC Press, Boca Raton, FL. 401–436.

Wang, G.J., Tseng, H.W., Chou, C.J., Tsai, T.H., Chen, C.T., & Lu, M.K. (2003). The vasorelaxation of Antrodia camphorata mycelia: involvement of endothelial Ca(2+)-NO-cGMP pathway. *Life Science*, 73, 2769-2783.

Williams, A.T., & Burk, R.F. (1990). Carbon tetrachloride hepatotoxicity: an example of free radical-mediated injury. *Seminars in Liver Disease, 10*, 279–284.

Wink, D.A., Cook, J.A., Pacelli, R., DeGraff, W., Gamson, J., Liebmann, J., Krishna, M.C., & Mitchell, J.B. (1996). The effect of various nitric oxide-donor agents on hydrogen peroxide-mediated toxicity: a direct correlation between nitric oxide formation and protection. *Archives of Biochemistry and Biophysics, 331*, 241–248.

Yen, G.C., Lai, H.H., & Chou, H.Y. (2001). Nitric oxide-scavenging and antioxidant effects of *Uraria crinita* root. *Food Chemistry, 74,* 471—478.

Yeh, C.T., Rao, Y.K., Yao, C.J., Yeh, C.F., Li, C.H.,Chuang, S.E., Luong, J.H., Lai, G.M., & Tzeng, Y.M. (2009). Cytotoxic triterpenes from *Antrodia camphorata* and their mode of action in HT-29 human colon cancer cells. *Cancer Lett*er, *285,* 73-79.