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# *In vitro* protective effect of *Schisandra chinensis* extract against carbon tetrachloride–induced hepatotoxicity in common carp (*Cyprinus carpio*)

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**In the present study, *in vitro* hepatoprotective effect of *Schisandra chinensis* extract (SCE) was evaluated against carbon tetrachloride ($CCl_4$)–induced hepatotoxicity in common carp. SCE (100, 200, and 400 µg $ml^{-1}$) was added to the carp primary hepatocytes before (pre-treatment), after (post-treatment), and both before and after (pre and post-treatment) the exposure of the hepatocytes to 8 mM $CCl_4$ in the culture medium. Results showed that exposure of the primary cultured carp hepatocytes to 8 mM $CCl_4$ for 4 h caused cytotoxicity, manifested by loss of cell viability and significantly elevated levels of lactate dehydrogenase (LDH), glutamate oxalate transaminase (GOT), glutamate pyruvate transaminase (GPT) and malondialdehyde (MDA), and significantly reduced activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the supernatant. Pre-treatment and pre and post-treatment of the hepatocytes with SCE significantly reduced the elevated levels of LDH, GOT, GPT and MDA; increased the reduced activities of SOD and GSH-Px and increased the cell viability in a dose-dependent manner. Post-treatment of the hepatocytes with SCE did not show significant effects on the tested parameters except GPT. The results suggest that SCE is a potent hepatoprotective agent that could protect fish hepatocytes against the acute injury and this ability might be attributed to its antioxidant potential. The results also imply that SCE can be potentially used for preventing rather than curing liver diseases in fish.**

**Key words:** Carp primary hepatocytes, hepatoprotection, antioxidant, *Schisandra chinensis*.

## INTRODUCTION

In aquatic environment, fish are directly exposed to various natural and synthetic chemicals originated from agricultural and industrial activities. Liver is prone to xenobiotic-induced injury because of its central role in xenobiotics metabolism, its portal location within the circulation, and its anatomic and physiologic structure (Sturgill and Lambert, 1997). Fish liver neoplasm due to chemical challenge has been frequently reported in polluted areas (Malins et al., 1988; Myers et al., 1991; Myers et al., 2003; Stehr et al., 1997; Koehler, 2004). The use of chemicals in aquaculture systems for various purposes is widely recognized, especially in intensive pond aquaculture system in Asia. The heavy use of prophylactic antibiotics in aquaculture has become a growing problem for human and animal health and for the environment (Cabello, 2006). An increasing number of

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chemicals have shown the potential to induce lesions in the liver of fish (Dixon et al., 1987; Webb et al., 2008; Braunbeck et al., 1990; Oulmi et al., 1995), antimicrobial agents are a common and important cause of hepatotoxicity (Thiim and Friedman, 2003). Oxytetracycline-induced liver injury has been reported in rainbow trout and Atlantic salmon (Brown and Desmond, 2002; Thiim and Friedman, 2003).

Recently, fish disease called hepatobiliary syndrome, with the symptoms of liver and gall bladder enlargement (up to 2 to 3 times of their original sizes) and colour changing, has been frequently reported in many cultured species and caused dramatic loss in China. Histological and biomedical investigations revealed the hepatocyte necrosis and increases in activities of serum glutamate oxalate transaminase (GOT) and glutamate pyruvate transaminase (GPT) in grass carp suffering from hepatobiliary syndrome (Liu et al., 2009). It is a non-infectious disease, pathogenic bacteria or viruses have not been isolated, and it was proposed that xenobiotic challenge due to drug abuse may be one of the important causes of the disease (Shi and Wei, 2010). So far, no effective methods have been found for the treatment of hepatobiliary syndrome, and much attention has been focused on the use of Chinese medicinal herbs to prevent and control this disease (Li et al., 2011).

$CCl_4$-induced hepatocyte damage is the best-characterized system of the xenobiotic-induced hepatotoxicity, it is frequently used to screen hepatoprotective agents including nutritional supplements and liver protective drugs (Rechnagel and Glende Jr, 1973), and it is also widely used for the study of hepatoprotective effects of drugs and herbal extracts in mammals (Ahsan et al., 2009). However, most of our understanding of $CCl_4$-induced hepatotoxicity remains confined to mammal models (Guillouzo, 1998), and data obtained in mammal cannot be extrapolated with certainty to the fish situation. Therefore, to screen the hepatoprotective Chinese medicinal herbs specific for liver disorder in fish, an *in vitro* model of $CCl_4$-induced hepatotoxicity in primary cultured carp hepatocytes was previously established in our laboratory, and it has been successfully used to evaluate the hepatoprotective and antioxidant effects of *Glycyrrhiza glabra* extract in fish (Yin et al., 2011). Primary cultured hepatocytes generally maintain many of their original differentiated *in vivo* characteristics and therefore facilitates extrapolation of the results to the *in vivo* situation (Pesonen and Andersson, 1997).

*Schisandra chinensis* is a traditional Chinese herb clinically prescribed for the treatment of various liver diseases in human beings because of its capability to protect the liver from injuries induced by various hepatotoxins (Zhu et al., 1999). Lignans including schizandrin A, B and C, schizandrol A and B, schizandrer A and B have been identified from the extract of *S. chinensis* and proven to have hepatoprotective effects against hepatic dysfunction induced by various chemical hepatotoxins in mammals (Xie et al., 2010). In fish, however, the hepatoprotective effect of *S. chinensis* has not been studied and its corresponding mechanisms have not been demonstrated yet. The present study is aimed at studying the effects of *S. chinensis* extract (SCE) on the function of fish hepatocytes using an *in vitro* model of $CCl_4$-induced hepatocyte injury and finding out whether it can be potentially used as a medicine for fish hepatobiliary syndrome.

## MATERIALS AND METHODS

### Chemicals

L-15 medium, ethylenediaminetetraacetic acid (EDTA), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), gentamicin sulphate, trypsin, insulin, streptomycin/penicillin and heparin were purchased from Sigma Company (St. Louis, Missouri, USA). Fetal bovine serum (FBS) and cell culture plates were ordered from Gibco Company (USA). $CCl_4$ was a product of National Pharmaceutical Group Chemical Reagent Co., Ltd., China. WST-1 was purchased from Beyotime Institute of Biotechnology, Haimen, China. *S. chinensis* extract (SCE) containing schizandrin A, schizandrin B and schizandrol A, was a commercial product obtained from Nantong Sihai Plant Extracts Co., Ltd., China.

### Fish

Common carp (*Cyprinus carpio*) was obtained from the Freshwater Fisheries Research Centre of Chinese Academy of Fishery Sciences, Wuxi, China. Fish were reared at 26°C in a recirculation system and fed *ad libitum* twice a day with commercial diets containing approximately 40% crude protein, 10% crude lipid, 10% ash and an energy content of 21 kJ $g^{-1}$ DM. They were about 6 months old at the start of the experiment, with an average weight of 150 g.

### Isolation and culture of hepatocytes

Fish hepatocytes were prepared according to the methods of Smeets et al. (1999) and Wan et al. (2004), with several modifications. Fish were anaesthetized in 0.05% tricaine methane sulphonate and sanitized with 70% alcohol, and blood was cleared from the caudal vein. An incision was made along the ventral midline from the vent to the gill isthmus. Two lateral incisions were made in the right ventral quadrant of the peritoneal cavity just anterior to the pelvic girdle and along the posterior margin of the gill operculum. The muscle and skin flap were removed, exposing the internal organs. Liver was then taken into a petri dish and washed with sterilized water and a $Ca^{2+}$- and $Mg^{2+}$-free buffer solution (pH 7.5) containing 0.145 M NaCl, 5.4 mM KCl, 5 mM EDTA, 1.1 mM $KH_2PO4$, 12 mM $NaHCO_3$, 3 mM $NaH_2PO_4$, 100 mM HEPES. The liver tissue was then minced into pieces and digested in a solution of 0.25% trypsin (1:20 w/v) for 30 min at room temperature. The mixture was further trypsinized on a shaker at 200 rpm for 5 min to obtain the cell suspension which was then filtered through a 70-mesh sieve. The cell suspension was then centrifuged at l00 *g* for 2 min and the cell pellet was washed 3 times with L-15 culture medium (pH 7.4) containing 14.3 mM $NaHCO_3$, 20 mM HEPES, 50 µg $ml^{-1}$ gentamicin sulphate, 1 mM insulin, 10 mM hydrocortisone, and 2% (v/v) FBS. The cell suspensions were pooled and centrifuged at 1000 rpm for 2 min, and the pellet was washed and resuspended in L-15 culture medium and counted. When viability was > 90% as assessed with Trypan blue exclusion, the cells were

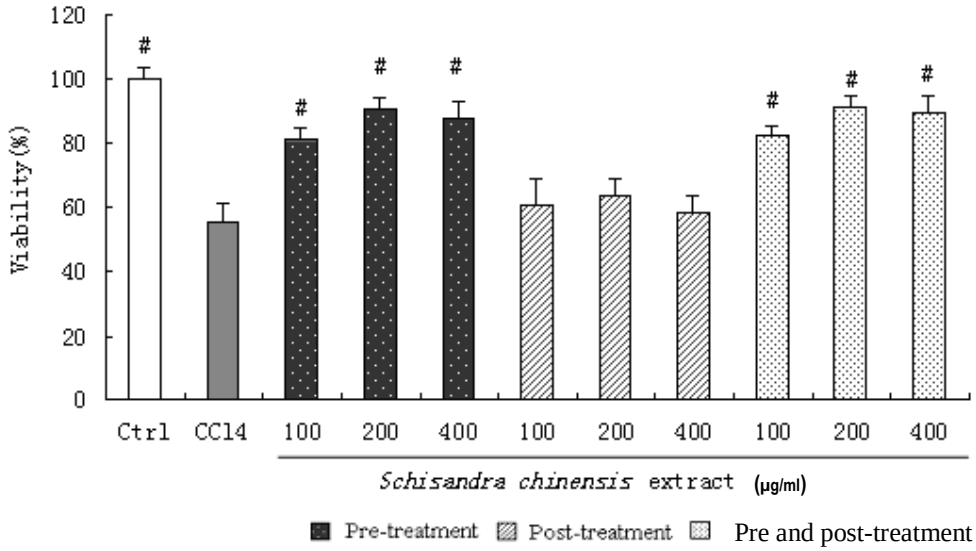


**Figure 1.** Effects of SCE on cell viability (percentage of control value) in $CCl_4$-treated primary hepatocytes. Values are expressed as mean ± SD (n = 4). $^{\#}P < 0.01$, compared with cells treated with $CCl_4$ only.

used for experiments. Hepatocytes were adjusted to a density of $2.5 \times 10^4$ $ml^{-1}$ viable cells and plated in 96-well microplates (200 µl $well^{-1}$) for viability assay or $2 \times 10^5$ $ml^{-1}$ viable cells and plated in 24-well microplates (600 µl $well^{-1}$) for biochemical assays. The cells were cultured in L-15 culture medium supplemented with 1% streptomycin/penicillin and 10% FBS and kept for 24 h at 27°C under 5% $CO_2$ before the following experiments were conducted.

***Treatments of hepatocytes with SCE***

The hepatoprotective effect of SCE was investigated using an *in vitro* model of $CCl_4$-induced hepatocellular injury. After 24 h incubation, the cells were treated under the following three separate conditions:

1. Pre-treatment: The cells were pre-incubated with 0, 100, 200 and 400 µg $ml^{-1}$ of SCE for 4 h before $CCl_4$ was added at a final concentration of 8 mM, the cells were then incubated with $CCl_4$ for 4 h.
2. Post-treatment: The cells were first incubated with $CCl_4$ at a concentration of 8 mM for 4 h and then SCE was added at concentrations of 0, 100, 200 and 400 µg $ml^{-1}$. The cells were then incubated with SCE for 4 h.
3. Pre and post-treatment: The cells were first pre-incubated with 0, 100, 200 and 400 µg $ml^{-1}$ of SCE for 4 h, then $CCl_4$ was added at a final concentration of 8 mM, after 4 h incubation with $CCl_4$, the cells were further treated with SCE at concentrations of 0, 100, 200 and 400 µg $ml^{-1}$ for another 4 h.

For each set of conditions, four experiments were performed. Control (without adding $CCl_4$ and SCE), $CCl_4$ treatment and 3 concentrations of SCE treatment were set, each treatment was performed in quadruplicate. Before SCE or $CCl_4$ were added, the old medium should be completely removed and replaced with fresh medium containing SCE or $CCl_4$. At the end of each set of experiment, a 0.5 ml aliquot of supernatants from each individual well was collected in a 1.5 ml tube, centrifuged and stored at -20°C for various assays mentioned.

***Parameter analysis***

Viability of hepatocytes treated with SCE was measured using the WST-1 cell proliferation and cytotoxicity assay kit in accordance with the manufacturer's instructions. Briefly, $5 \times 10^3$ cells were cultured in 96-well plate, after pre-treatment, post-treatment or pre- and post-treatment of the cells with SCE, 10 µl WST-1 was added to each well and the cells were incubated for an additional 2 h. The plate was shaken gently for 1 min before the absorbance of samples was measured under a wavelength of 450 nm using a microplate reader and the results were compared as percentages of control group. Lactate dehydrogenase (LDH), GPT, GOT, glutathione peroxidase (GSH-Px), superoxide dismutase (Donato et al., 2001) and malondialdehyde (MDA) in the supernatants were measured in a spectrophotometer (723C, Shanghai) using spectrophotometric diagnostic kits obtained from Nanjing Jiancheng Bioengineering Research Institute (Shen et al., 2009).

**Statistics**

The statistical analysis were performed with statistical package for social sciences (SPSS) software by one-way analysis of variance (ANOVA), followed by Tukey multiple comparison. $^{*}P < 0.05$; $^{\#}P < 0.01$ were used as the criterion for significance.

## RESULTS

### Effects of SCE on cell viability in hepatocytes exposed to $CCl_4$

Cultured hepatocytes treated with $CCl_4$ showed a significant reduction of cell viability compared to the control (Figure 1). Pre-treatment and pre and post- treatment of the hepatocytes with SCE at all the three concentrations

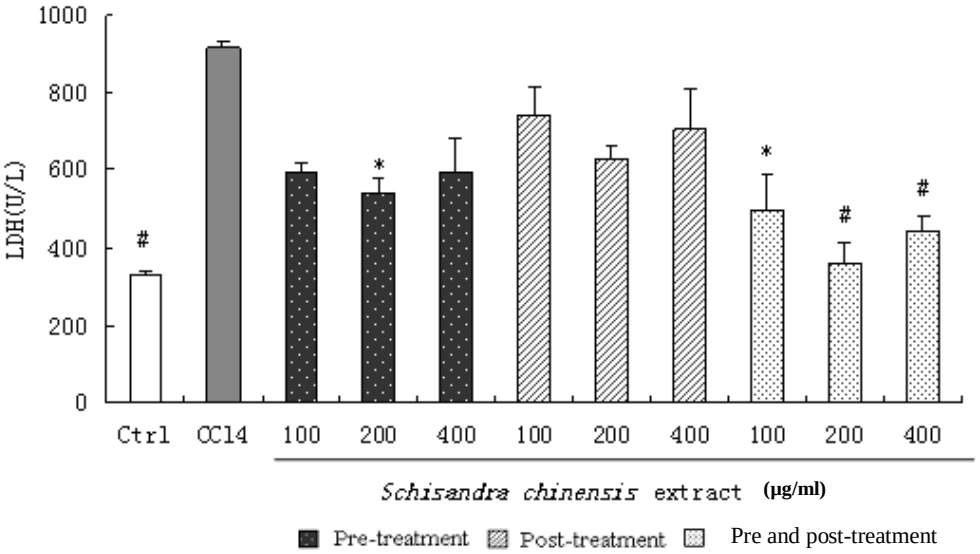


**Figure 2.** Effect of SCE on lactate dehydrogenase (LDH) in $CCl_4$-treated primary hepatocytes. Values are expressed as mean ± SD (n = 4). *$P$ < 0.05; #$P$ < 0.01, compared with cells treated with $CCl_4$ only.

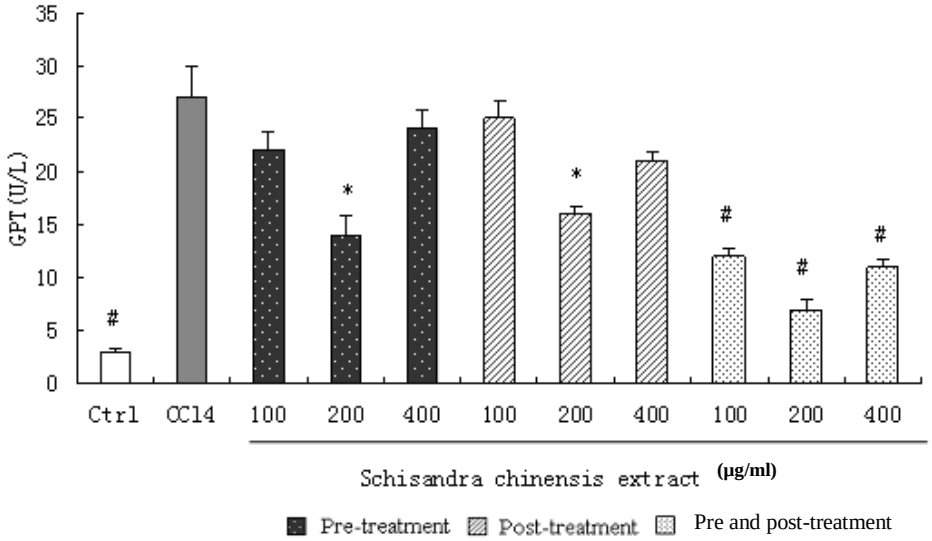


**Figure 3.** Effect of SCE on glutamate pyruvate transaminase (GPT) in $CCl_4$-treated primary hepatocytes. Values are expressed as mean ± SD (n = 4). *$P$ < 0.05; #$P$ < 0.01, compared with cells treated with $CCl_4$ only.

(100, 200 and 400 µg ml-1) significantly enhanced the cell viability ($P$ < 0.01). No significant difference was observed when the cells were post-treated with SCE.

### Effects of SCE on LDH, GPT and GOT activities in hepatocytes exposed to $CCl_4$

Cultured hepatocytes exposed to $CCl_4$ showed a 3-fold increase of LDH (Figure 2), a 9-fold increase of GPT (Figure 3) and a 6-fold increase of GOT (Figure 4) in the culture medium. Levels of all marker enzymes (LDH, GPT and GOT) increased significantly after the exposure of the hepatocytes to $CCl_4$, as compared to the control. SCE pre-treatment (200 µg $ml^{-1}$) and pre and post-treatment (100, 200 and 400 µg $ml^{-1}$) of the hepatocytes caused significant decreases in the activities of LDH, GPT and GOT (Figures 2 to 4). Dose-dependent effects were observed, pre and post-treatment with 200 µg $ml^{-1}$ of SCE caused the most significant effects ($P$ < 0.01) to reduce

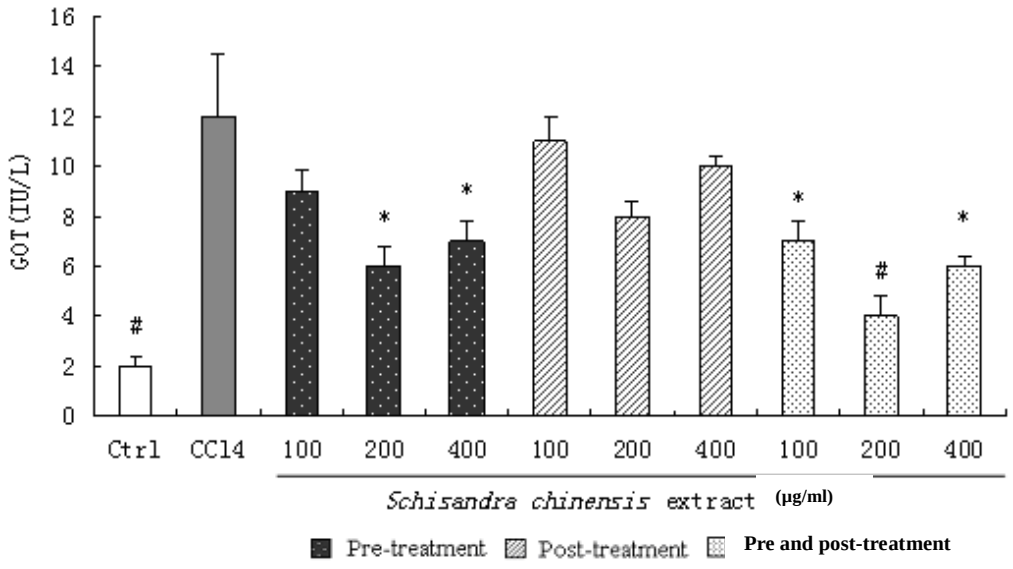


**Figure 4.** Effect of SCE on glutamate oxalate transaminase (GOT) in $CCl_4$-treated primary hepatocytes. Values are expressed as mean ± SD (n = 4). *$P$ < 0.05; #$P$ < 0.01, compared with cells treated with $CCl_4$ only.

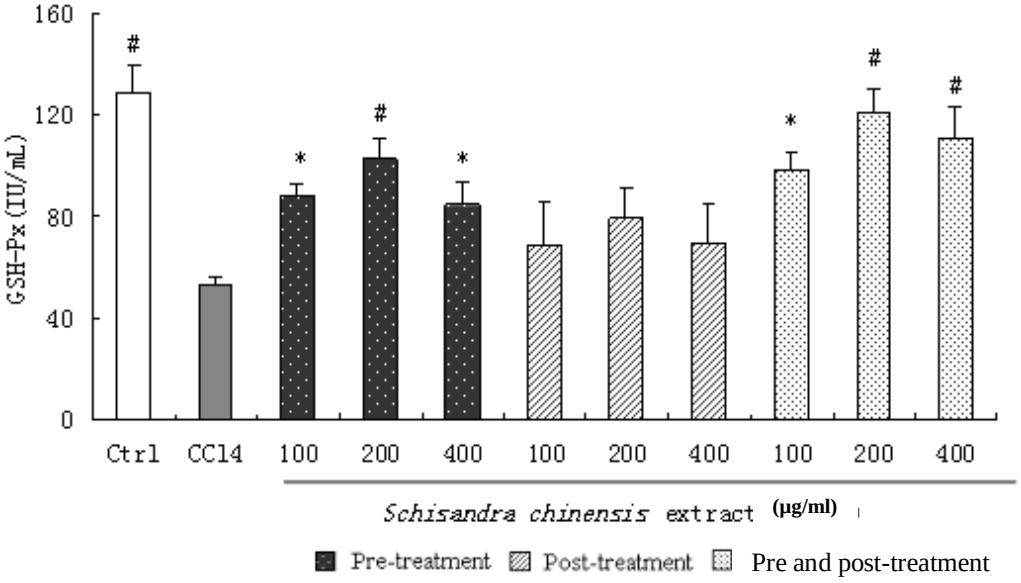


**Figure 5.** Effects of SCE on glutathione peroxidase (GSH-Px) in $CCl_4$-treated primary hepatocytes. Values are expressed as mean ± SD (n = 4). *$P$ < 0.05; #$P$ < 0.01, compared with cells treated with $CCl_4$ only.

the levels of LDH, GPT and GOT. However, post-treatments with SCE did not show any effects on the LDH and GOT activities (Figures 2 and 4). In the case of GPT activity, post-treatment of the cells with 200 μg $ml^{-1}$ of SCE still gave a significant effect, while no effects were observed when the cells were post-treated with 100 and 400 μg $ml^{-1}$ of SCE (Figure 3).

### Effects of SCE on GSH-PX and SOD activities in hepatocytes exposed to $CCl_4$

The activities of GSH-Px (Figure 5) and SOD (Figure 6) were significantly decreased when hepatocytes were treated with $CCl_4$, as compared to the control. Pre-treatment and pre and post-treatment of the hepatocytes

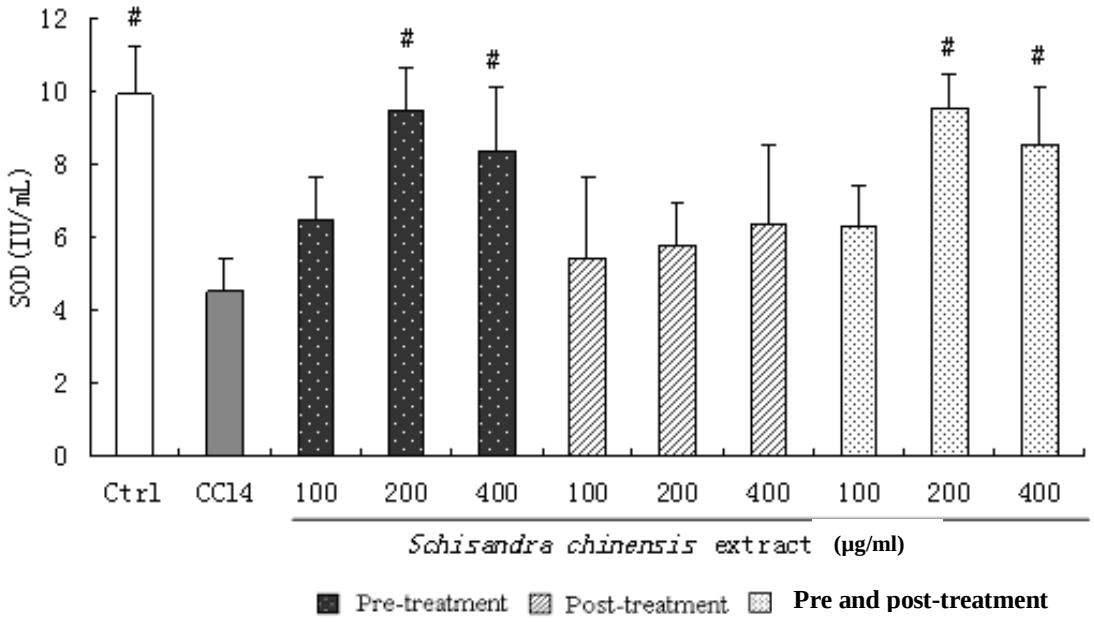


**Figure 6.** Effects of SCE on superoxide dismutase (SOD) in $CCl_4$-treated primary hepatocytes. Values are expressed as mean ± SD (n = 4). $^{\#}P < 0.01$, compared with cells treated with $CCl_4$ only.

with SCE restored the activity of GSH-Px in all the tested three concentrations (Figure 5), while restoration of SOD activity was observed only when the hepatocytes were pre-treated or pre and post-treated with SCE at 200 and 400 μg $ml^{-1}$ (Figure 6). Post-treatment of the hepatocytes with SCE did not show any effects on the GSH-Px and SOD activities (Figures 5 and 6).

### Effects of SCE on $CCl_4$-induced lipid peroxidation

Cultured hepatocytes treated with $CCl_4$ showed a 2.5-fold increase in the amount of MDA released into the medium (Figure 7). Pre-treating and pre and post-treating the cells with SCE at 200 and 400 μg $ml^{-1}$ significantly inhibited MDA formation, post-treating the cells with SCE did not show any effects on the MDA content (Figure 7).

## DISCUSSION

Hepatotoxicity induced by $CCl_4$ is a commonly used model for the screening of hepatoprotective drugs (Gilani and Janbaz, 1995). The biochemical mechanism involved in the development of $CCl_4$ hepatotoxicity has long been investigated: it is now generally believed that the formation of reactive trichloromethyl radicals ($\cdot CCl_3$) from $CCl_4$ by CYP 450 is a crucial factor in the pathogenesis of $CCl_4$ hepatotoxicity (Ip and Ko, 1996). In the presence of oxygen, $\cdot CCl_3$ is quickly transformed into trichloromethyl peroxyl radical ($CCl_3O_2\cdot$), $CCl_3O_2\cdot$ binds covalently to cellular proteins or lipids, and initiates the lipid peroxidation in the cellular membrane (Levine and Reinhardt, 1983) resulting in the leakage of cellular enzymes (LDH, GPT and GOT) and finally cell apoptosis and necrosis (Manibusan et al., 2007). Therefore, cell viability and leakage of cytosolic enzymes (LDH, GPT and GOT) have been frequently used to assess the $CCl_4$ hepatotoxicity (Visen et al., 1998).

In the present study, the loss of cell viability and the significant elevated activities of LDH, GPT and GOT in the supernatants of the $CCl_4$-treated hepatocytes indicated the cellular leakage or hepatocyte damage. Pre-treatment or pre and post-treatment of the hepatocytes with SCE significantly increased the cell viability and decreased the activities of LDH, GPT and GOT, indicating that SCE could maintain the functional integrity of the hepatocyte membrane, protect the hepatocytes against $CCl_4$-mediated toxicity. Our results are consistent with studies on mammals both *in vitro* and *in vivo* that SCE was effective in reducing the increased activities of GPT, GOT and LDH induced by $CCl_4$ (Hancke et al., 1999; Qi et al., 2009).

MDA, a decomposition product of lipid hydroperoxides (Gutteridge, 1995) is widely used as marker of lipid peroxidation (Mansour, 2000), its elevated level could reflect the degree of lipid peroxidation injury in hepatocytes (Hu et al., 2001). In this study, MDA levels in the supernatant of $CCl_4$ treated hepatocytes were significantly elevated, pre-treatment or pre and post-treatment of the hepatocytes with SCE (200 and 400 μg $ml^{-1}$) significantly suppressed the elevation of MDA caused by $CCl_4$, indicating the anti-lipid peroxidation effect of SCE.

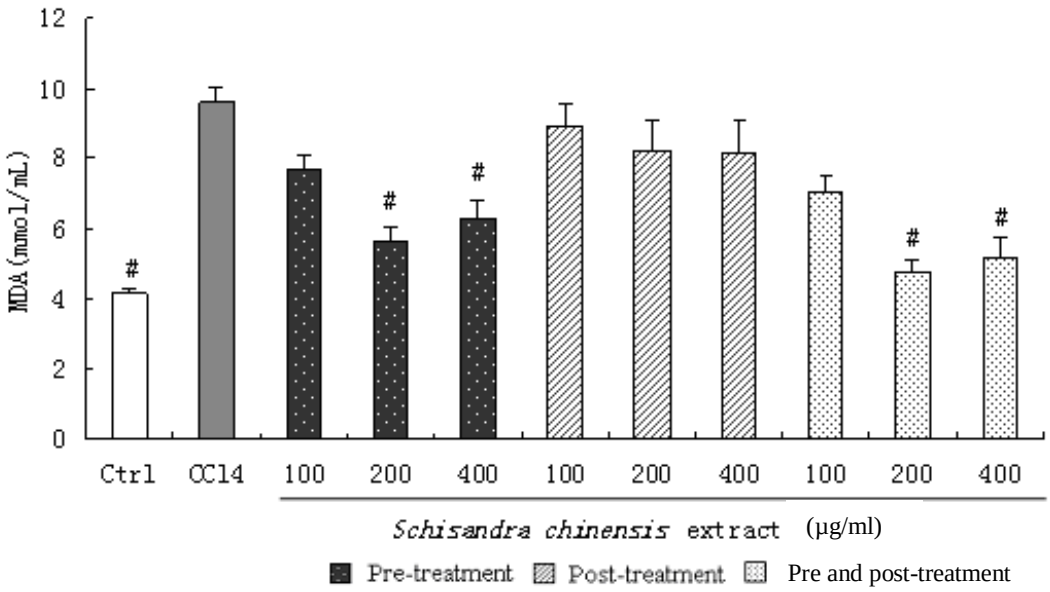


**Figure 7.** Effects of SCE on malondialdehyde (MDA) in $CCl_4$-treated primary hepatocytes. Values are expressed as mean ± SD (n = 4). $^{\#}P < 0.01$, compared with cells treated with $CCl_4$ only.

Antioxidant property is claimed to be one of the mechanisms of hepatoprotective drugs (Recknagel, 1967). GSH-Px and SOD are two important antioxidant enzymes involved in enzymatic antioxidant defence mechanisms (Kalayci et al., 2005). It has been suggested that the lipid peroxidases generated after $CCl_4$ treatment is eliminated by GSH-Px in the presence of glutathione, thus curbing the propagation of lipid peroxidation (Koneri et al., 2008). The significant decreases of GSH-Px and SOD activities in $CCl_4$-treated hepatocytes in this study may partly explain the 2.5-fold elevation of MDA, while pre-treatment or pre- and post-treatment with SCE restored the GSH-Px and SOD activities (Figures 5 and 6), which may contribute to the suppressed lipid peroxidation as evidenced by the suppressed formation of MDA. The antioxidant effect of *S. chinensis* is attributed to its lignan constituents such as schisandrin B and schisanhenol (Hancke et al., 1999), which increased superoxide dismutase and catalase activities in rat liver cytosol (Johnston and Santillo, 2002), inhibited the lipid peroxidation measured by means of MDA formation induced by iron/cysteine in rat liver microsomes (Hahn, 2002).

Comparing the pre-treatment with the post-treatment regimen, we found that pre-treatment with SCE showed the protective effect against $CCl_4$-mediated toxicity, while post-treatment did not show significant effects on all the tested parameters except GPT; this may suggest that SCE can be potentially used for preventing rather than curing liver diseases in fish. The present findings demonstrated the hepatoprotective effect of SCE against hepatocyte damage induced by $CCl_4$ in fish. The hepatoprotective activity of SCE may be attributed to the enhancement of the hepatic antioxidant system. Further *in vivo* studies may provide better understanding of SCE as a hepatoprotective agent potential for the prevention of hepatobiliary syndrome in fish.

## ACKNOWLEDGMENTS

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