

Swertiamarin attenuates carbon tetrachloride (CCl₄)-induced liver injury and inflammation in rats by regulating the TLR4 signaling pathway

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The aim of the present study is to illustrate the effects of swertiamarin (STM), a natural iridoid from herbal medicines, on hepatic inflammation induced by carbon tetrachloride (CCl₄) in rats. Male Sprague Dawley rats were exposed to CCl₄ with or without STM co-administration for 8 weeks. Our results revealed that STM administration (100 and 200 mg/kg b.w.) significantly attenuated inflammation in livers of CCl₄-treated rats. STM remarkably reduced the production of interleukin-1 β (IL-1 β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), macrophage inflammatory protein-1 α (MIP-1 α), and monocyte chemoattractant protein-1 (MCP-1) in liver tissue of CCl₄-treated rats. In addition, STM treatment downregulated connective tissue growth factor (CTGF) and ser307pIRS-1 expression, which was induced by CCl₄ exposure. In the process of exploring the anti-inflammatory mechanisms of STM action, we demonstrated that STM significantly inhibited Toll-like receptor 4 (TLR4) and nuclear factor kappa B (NF- κ B) p65 expression in the liver. In conclusion, these results suggested that the inhibition of CCl₄-induced inflammation by STM was, at least in part, due to its regulation of the TLR4 /NF- κ B signaling pathway.

Keywords: Swertiamarin/effects. Carbon tetrachloride. Inflammation. TLR4. NF- κ B.

INTRODUCTION

Swertiamarin (STM) is a bitter secoiridoid glycoside isolated from gentianaceae medicinal plants exerting notable hepatoprotective effects (Jaishree, Badami 2010; Zhang *et al.*, 2015). In addition, we have previously demonstrated that STM could alleviate oxidative stress in CCl₄-induced hepatotoxicity (Wu *et al.*, 2017). However, the underlying molecular mechanisms for its hepatoprotective effects have not been fully elucidated.

There are many molecular events involved in the pathogenesis of the liver injury such as oxidative stress, inflammation, and immune reactions. Among them, tissue damage associated with hepatic inflammation can be mediated by the proinflammatory cytokines such as inducible nitric oxide synthase, interleukin-1 β (IL-1 β) and tumor necrosis factor alpha (TNF- α) (Ma *et al.*,

2014). These cytokines can stimulate the Kupffer cells to release various inflammatory mediators and free radicals (Decker 1990). The activation of inflammatory cells, including Kupffer cells, is a crucial step for activating hepatic stellate cells. Toll-like receptors (TLRs) have been reported to play an essential role in the activation of innate immunity (Pestka, Zhou, 2006). In response to stimulation of external risk factors, tissues engage the innate immune response and up-regulate TLR4 expression. Activated TLR4 will then stimulate further immune responses. Various signaling molecules have been shown to regulate the TLR pathway to modulate innate immune responses (Fang *et al.*, 2016). The nuclear factor kappa B (NF- κ B) is one of the most ubiquitous transcription factors modulating the immune response to infection or stimuli (Tornatore *et al.*, 2012). Expression of the proinflammatory cytokines is modulated by NF- κ B. Therefore, NF- κ B and TLRs have attracted interest as targets for the treatment of inflammatory liver damage (Shin *et al.*, 2013; Ma *et al.*, 2015).

The present study aimed to explore the anti-

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inflammatory effects and mechanisms of STM on CCl₄-induced liver injury.

MATERIAL AND METHODS

Chemicals and reagents

Swertiamarin (purity>98%, dissolved in 1% Tween-20 saline) was provided by Xi'an Jiatian Biotechnology Co., Ltd (Xi'an, China). Carbon tetrachloride was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The levels of aspartate aminotransferase (ALT) and alanine aminotransferase (AST) were determined by using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, China). The ELISA kits for IL-1 β , interleukin-6 (IL-6), TNF- α , macrophage inflammatory protein-1 α (MIP-1 α), and monocyte chemoattractant protein-1 (MCP-1) were purchased from Neobioscience (Beijing, China). Antibodies against connective tissue growth factor (CTGF) (Boster, PB0570), TLR4 (Boster, BA1717), NF- κ B p65 (Santa Cruz, sc-1173), ser307pIRS-1 (Boster, P00268), GAPDH (Boster, BA2913), and Histone H3 (Santa Cruz, sc-166574) were used in this study.

Animals

This study was carried out in strict accordance with the guideline of the Council on Animal Care of Academia Sinica. The protocol was approved by the Ethical Committee on Animal Experimentation of Pua Hospital, Tongji Medical College, Huazhong University of Science and Technology, China. Adult male Sprague Dawley rats weighing 250-280 g were obtained from the Center of Experimental Animals of Hubei Province (Wuhan, China). All animals were kept under the same laboratory conditions of temperature (25 \pm 2 $^{\circ}$ C) and lighting (12:12 h light: dark cycle) and were given free access to standard laboratory chow and tap water. All rats were allowed to acclimatize for 1 week before the experiment.

Experimental design

The animals were randomly assigned to five experimental groups (n=12): (1) Control group, rats were given 1% Tween-20 saline by gavage once per day for 8 consecutive weeks with co-administration of vehicle (peanut oil, solvent of carbon tetrachloride, 0.3 mL/100 g, subcutaneously [s. c.] twice a week); (2) STM 200 mg/kg group, rats were given STM dissolved in 1% Tween-20 saline (200 mg/kg body weight [b.w.]) by gavage once

per day for 8 consecutive weeks with co-administration of vehicle (peanut oil, solvent of carbon tetrachloride, 0.3 mL/100 g, s. c. twice a week); (3) CCl₄ group, rats were given 1% Tween-20 saline by gavage once per day for 8 consecutive weeks with co-administration of 40% CCl₄ mixed peanut oil solution (0.3 mL/100 g, s. c. twice a week); (4) CCl₄+STM 100 mg/kg group, rats were treated with STM dissolved in 1% Tween-20 saline (100 mg/kg b.w.) by gavage once per day for 8 consecutive weeks with co-administration of 40% CCl₄ mixed peanut oil solution (0.3 mL/100 g, s. c. twice a week); (5) CCl₄+STM 200 mg/kg group, rats were treated with STM dissolved in 1% Tween-20 saline (200 mg/kg B.W.) by gavage once per day for 8 consecutive weeks with co-administration of 40% CCl₄ mixed peanut oil solution (0.3 mL/100 g, s. c. twice a week). The rats were sacrificed at the end of 8 weeks and 24 h after the last dose of CCl₄. Blood and liver samples were collected for further analysis.

Histological examination

Liver samples were derived from the central part of the right large lobe of the rats. For hematoxylin and eosin staining, the liver tissues were fixed with 10% formalin for 24 h, and then washed with tap water, dehydrated, and embedded in paraffin. Each slide was subjected to histological assessment.

Measurement of serum aminotransferase activities

The activities of ALT and AST in serum were determined spectrophotometrically using commercial diagnostic kits (Jiancheng Institute of Biotechnology, Nanjing, China).

Assay of IL-1 β , IL-6, TNF- α , MIP-1 α , and MCP-1 levels in liver tissue

The IL-1 β , IL-6, TNF- α , MIP-1 α and MCP-1 levels in liver tissue were determined using the respective ELISA kits (Neobioscience) according to the instructions of manufacturers.

Western blot analysis

The whole liver lysate was prepared to evaluate the expression level of CTGF and TLR4. The nuclear extracts of liver tissue were obtained to evaluate the expression level of nucleus NF- κ B p65 by using a nuclear/cytoplasmic isolation kit (Beyotime Institute of Biotechnology, Beijing,

China). The protein concentration was determined using the bicinchoninic acid assay and samples were stored at -80 °C. An equal amount of protein per lane was separated by standard 10% SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane (Millipore). Then the membranes were blocked with Tris-buffered saline containing 5% nonfat milk at 4°C. After that, the membranes were respectively incubated overnight at 4°C in solution containing 0.1% Tween 20, 5% nonfat milk and the following primary antibodies: CTGF (1:500), TLR4 (1:500), NF-κB p65 (1:400); ser307pIRS-1 (1:400); GAPDH (1:20000); Histone H3 (1:1000). After three washes in Tris-buffered saline Tween 20, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies at room temperature. The protein bands were quantified using the Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD). GAPDH or Histone H3 was used as an internal control.

Statistical analysis

Data were expressed as mean ± S.D. The significant differences between groups were assessed with SPSS version 13.0. The differences between group means were calculated by one-way ANOVA with LSD post hoc analysis. Difference was considered statistically significant when $p < 0.05$, and extremely significant when $p < 0.01$.

RESULTS AND DISCUSSION

STM alleviated CCl₄-induced liver tissue damage

The hepatic samples of the control and STM 200mg/kg groups presented normal architecture. However, liver tissues from rats intoxicated with CCl₄ showed extensive histopathological changes, characterized by severe hepatocytes degeneration/necrosis, fatty changes, and inflammatory cell infiltration and congestion. The incidence and severity of histopathological lesions in CCl₄+STM groups were less than those in the CCl₄ group (Figure 1).

STM reduced CCl₄-elevated serum ALT and AST activities

Repeated CCl₄ exposure showed elevated activities of serum ALT and AST. Treatment with STM obviously reduced serum ALT and AST activities as compared with the CCl₄ group ($p < 0.01$, Figure 2A-B), which indicated the restoration of liver function.

STM reduced the production of pro-inflammatory cytokines

The proinflammatory cytokines (IL-1β, IL-6, TNF-α, MIP-1α, and MCP-1) profile in liver shown in

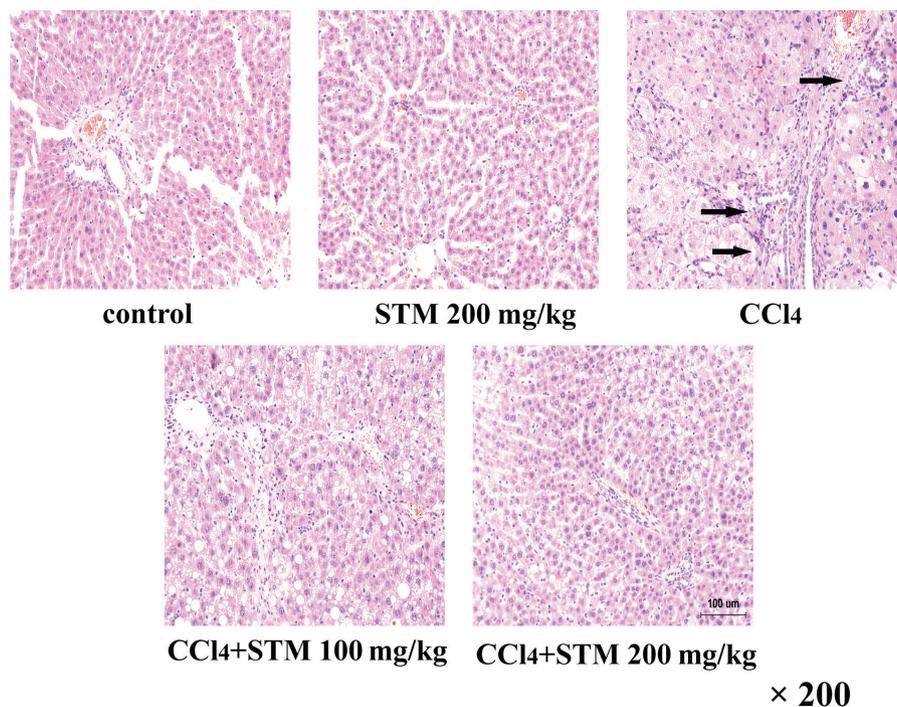


FIGURE 1 - STM alleviated CCl₄-induced histology changes in the liver. (A) Control group, (B) STM 200 mg/kg group, (C) CCl₄ group, (D) CCl₄+STM 100 mg/kg group, (E) CCl₄+STM 200 mg/kg group. Arrows represent the large amount of inflammatory cell infiltration. H&E staining, Magnification: 200×.

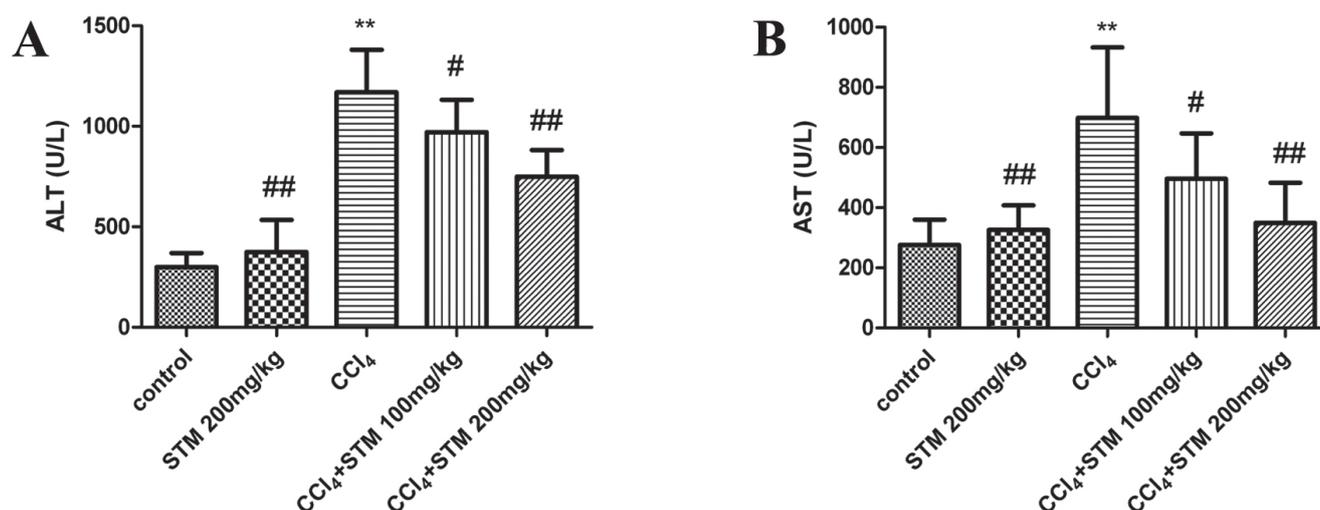


FIGURE 2 - STM improved CCl₄-induced changes in hepatic functional markers. (A) ALT activity; (B) AST activity. Data are represented as means \pm S.D. for 7-12 animals per group. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus CCl₄, ## $p < 0.01$ versus CCl₄ by one-way ANOVA and LSD post hoc test.

Figure 3 showed a significant increase in proinflammatory cytokines in the CCl₄-intoxicated rats as compared with the control rats. The IL-1 β , IL-6, TNF- α , MIP-1 α , and MCP-1 content in liver tissue of the STM-treated rats was significantly lower than that in the CCl₄ group, suggesting the alleviation of liver inflammation with STM treatment. Note: treatment with STM alone did not have an obvious influence on the cytokine profile.

STM downregulated CTGF protein expression

The effect of STM on CTGF protein level in liver was determined. Western blotting showed that CTGF protein expression in liver tissue was significantly increased in the CCl₄ group as compared with the control group ($p < 0.05$). STM treatment down-regulated CTGF protein level notably in liver tissue as compared with the CCl₄ group (Figure 4).

STM regulated protein expression in the TLR4 signaling pathway

The effect of STM on the TLR4 signaling pathway activation was examined. We assessed the activation of the signaling molecules, including TLR4 and NF- κ B p65. Western blotting showed that cytoplasmic TLR4 and nuclear NF- κ B p65 protein expression in liver tissue was significantly induced in the CCl₄ group as compared with the control group ($p < 0.01$). STM treatment markedly inhibited the CCl₄-induced increase of TLR4 and NF- κ B p65 protein expression in liver tissue. The results are shown in Figure 4.

STM downregulated pIRS-1

The expression level of phosphorylated ser-307 IRS-1 in a liver sample was determined with western blotting. The ser307pIRS-1 expression in liver tissue was significantly elevated in the CCl₄ group compared with the control group ($p < 0.05$). STM treatment (200 mg/kg b.w.) downregulated the ser307pIRS-1 level notably in liver in comparison with the CCl₄ group (Figure 5).

In this article we showed that STM, a natural iridoid compound, was instrumental in attenuating liver injury in rats induced by a repeated CCl₄ intoxication. The doses of STM (100 and 200 mg/kg b.w.) were based on the previously published articles on experimental liver injury (Jaishree, Badami 2010; Zhang *et al.*, 2015; Chen *et al.*, 2017). In the present study, a repeated CCl₄ exposure resulted in a significant increase in serum ALT and AST levels, biomarkers of liver injury. In addition, severe liver lesion and inflammatory response were also triggered by CCl₄. All of the aforementioned pathological changes were significantly improved with STM treatment. Additionally, cytoplasmic TLR4 and nuclear accumulation of the NF- κ B p65 in the livers of CCl₄-intoxicated rats were significantly decreased by the administration of STM, suggesting a possible mechanism of action.

Liver inflammation is a common response to various types of chronic liver injury. In the initial stages of inflammation, the hepatocytes, Kupffer cells, platelets, and leukocytes are activated to produce ROS and inflammatory mediators such as platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), CTGF, and TNF- α . These factors probably act as paracrine mediators

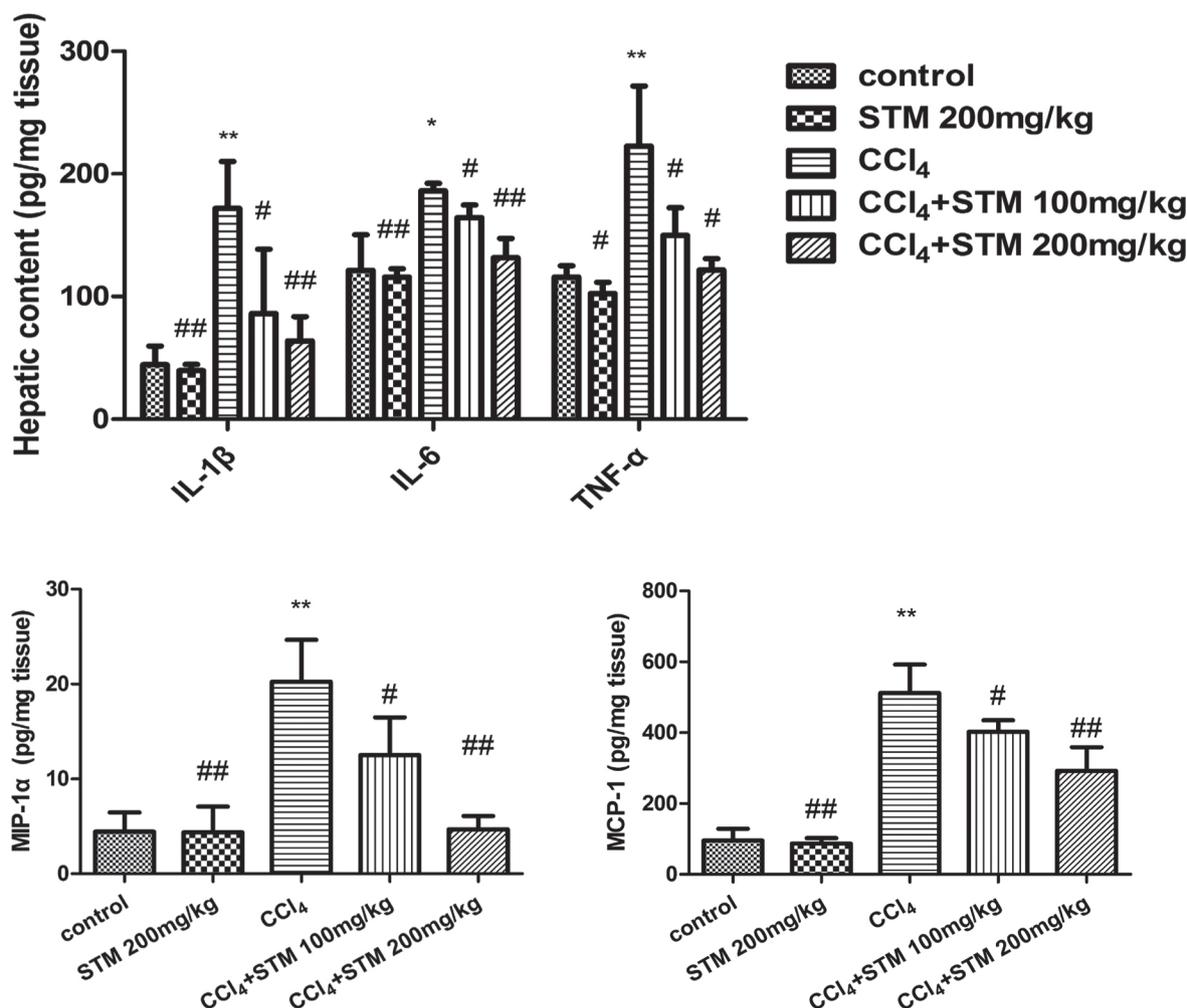


FIGURE 3 - STM inhibited the hepatic production of IL-1 β , IL-6, TNF- α , MIP-1 α , and MCP-1. The contents of IL-1 β , IL-6, TNF- α , MIP-1 α , and MCP-1 in liver were significantly increased following 8 weeks of chronic CCl₄ exposure compared with the controls. STM (100 and 200 mg/kg) treatment reduced the production of IL-1 β , IL-6, TNF- α , MIP-1 α , and MCP-1 notably compared with the CCl₄ group. Data are represented as means \pm S.D. for 7-12 animals per group. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus CCl₄, ## $p < 0.01$ versus CCl₄ by one-way ANOVA and LSD post hoc test.

to activate quiescent HSCs that are localized in the perisinusoidal space, resulting in an abnormal quantity and composition of extracellular matrix, which in turn leads to hepatitis, liver fibrosis, and cirrhosis (Wu, Zern, 2000). Thus, it is important to suppress hepatic inflammation in the early stages of liver fibrosis.

Cytokines/chemokines may promote inflammatory development via a wide variety of cascade events. IL-6 release can relate to acute phase of liver lesions associated with inflammation and may induce the release of other proinflammatory cytokines (Liang *et al.*, 2014). Considerable evidence suggests that IL-1 β and TNF- α contribute to the pathogenesis of liver inflammatory diseases by activating the NF- κ B signaling pathway (Muriel, 2009). In addition, it has been reported that acute hepatotoxicity with CCl₄ leads to NF- κ B activation and

very high expression of IL-1 β , IL-6, and TNF- α (Reyes-Gordillo *et al.*, 2007). In addition, chronic CCl₄ treatment could also induce a severe degree of liver inflammation and necrosis evidenced by an increase in the mRNA levels of various proinflammatory cytokines and chemokines such as TNF- α , IL-1 β , MIP-1 α , MIP-2, and MCP-1 (Mukhopadhyay *et al.*, 2014). These cytokines are also valuable indicators of hepatic inflammation. In our study, STM treatment significantly inhibited these cytokines (IL-1 β , IL-6, TNF- α , MIP-1 α , and MCP-1) induced by CCl₄ that were consistent with relieved liver injury.

Hepatic fibrogenesis depends on preexistent and continuous liver injury, in part due to inflammatory cell infiltration (Gao, Jeong, Tian, 2008). CTGF is a matricellular protein that participates in critical processes including differentiation, development, tumor growth, and

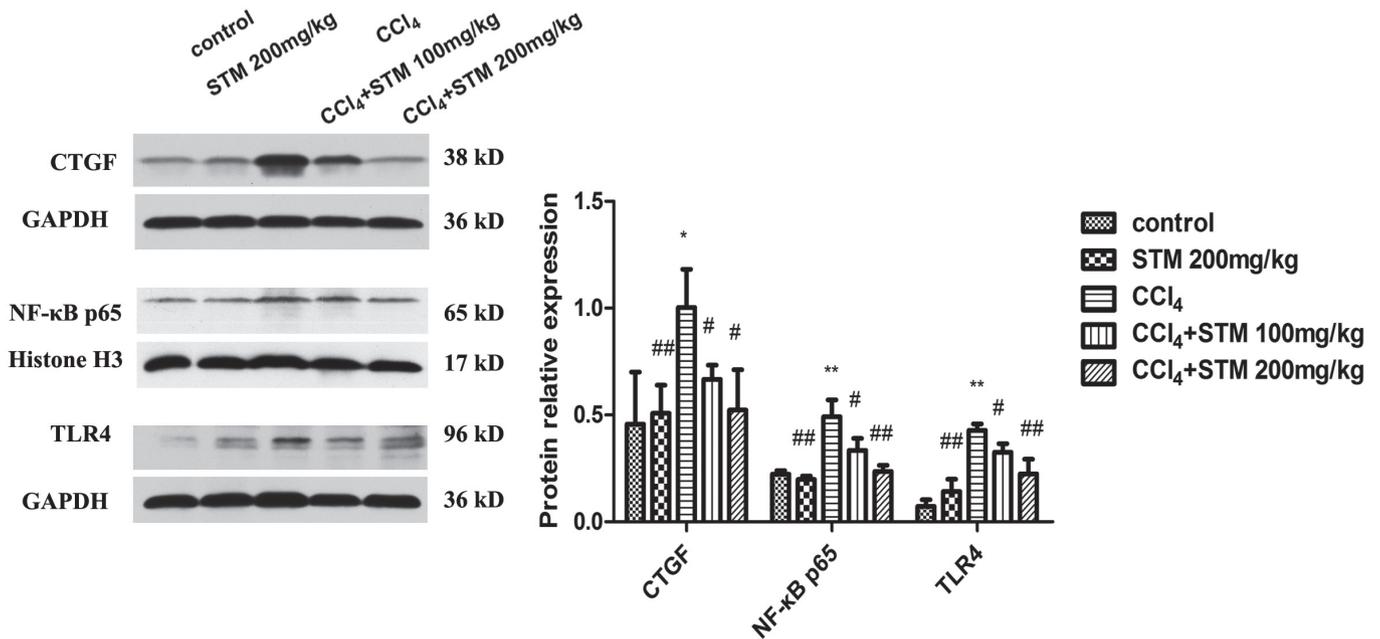


FIGURE 4 - STM repressed the protein expression of CTGF, TLR4 and NF-κB p65 in the liver. The CTGF protein level of rats in CCl₄+STM 100 mg/kg and CCl₄+STM 200 mg/kg groups were downregulated significantly compared with the CCl₄ group. The protein expression of TLR4 and NF-κB p65 of rats in CCl₄+STM 100 mg/kg and CCl₄+STM 200 mg/kg groups were significantly lower than that in the CCl₄ group. Data are represented as means ± S.D for 3-4 animals per group. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus CCl₄, ## $p < 0.01$ versus CCl₄ by one-way ANOVA and LSD post hoc test.

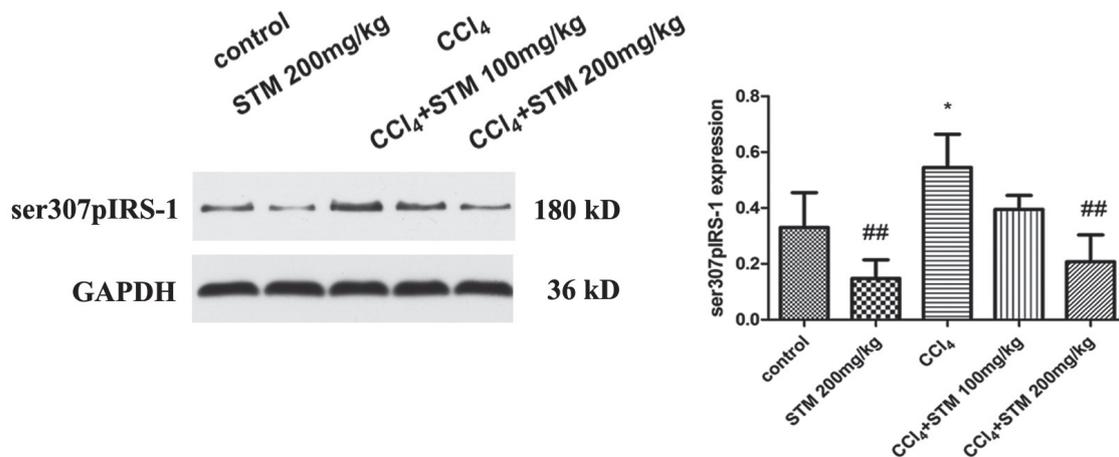


FIGURE 5 - STM downregulated pIRS-1. The expression level of the ser307pIRS-1 in liver tissue was significantly elevated in the CCl₄ group as compared with the control group ($p < 0.05$). STM (200 mg/kg b.w.) treatment downregulated the ser307pIRS-1 level notably in liver as compared with the CCl₄ group ($p < 0.01$). Data are represented as means ± S.D. for 3-4 animals per group. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus CCl₄, ## $p < 0.01$ versus CCl₄ by one-way ANOVA and LSD post hoc test.

wound healing (Brigstock, 1999). CTGF has been shown to be a pivotal mediator of hepatic fibrogenesis (Tong *et al.*, 2009). The expression of CTGF is obviously elevated in livers from rodents subjected to bile duct ligation or exposure to CCl₄ (Williams *et al.*, 2000; Sedlaczek *et al.*, 2001). Our results showed that STM significantly

downregulated CTGF expression, which was induced by CCl₄, contributing to the restrictions on fibrogenesis and inflammatory reaction in the liver.

CCl₄ intoxication can activate numerous intracellular signaling pathways (such as MAPK, TGF-β1), which may converge on NF-κB (Ma *et al.*,

2011; Wang *et al.*, 2013). Activation of NF- κ B in liver cells results in the recruitment of inflammatory cytokines/mediators, thus inducing fibrosis (Luedde, Schwabe, 2011). In addition, many studies from mouse and human had indicated that TLRs are central mediators of the inflammatory response and crucial links between inflammation and fibrosis in chronic liver diseases (Schwabe, Seki, Brenner, 2006; Berzsenyi *et al.*, 2011). TLR4 exhibits a fundamental role in managing innate immunity activation and regulates the recruitment of cytokines that are necessary for the progression of inflammation (Wei *et al.*, 2015). TLR4 is also strongly associated with the progression of liver fibrosis (Aoyama, Paik, Seki, 2010). Specifically, modulation of TGF- β signaling by a TLR4-MyD88-NF- κ B axis provides a novel link between proinflammatory and profibrogenic signals (Seki *et al.*, 2007). The present findings revealed that high levels of NF- κ B and TLR4 in liver cells of CCl₄-treated rats reflected liver impairments that are associated with inflammation. Interestingly, these two proinflammatory genes were downregulated by administration of STM. As a result, we postulated that the molecular mechanism against CCl₄-triggered hepatotoxication is linked to STM-mediated inactivation of the TLR4/NF- κ B pathway, in which the benefit contributes to synergistic roles of attenuating immunotoxicity and inflammatory response in CCl₄-lesioned liver tissue, further improving liver functions.

CCl₄ intoxication could induce diffuse hepatosteatosis in rats (Cetinkaya *et al.*, 2013). Excess free fatty acids are activators of various protein kinases that lead to Ser 307 phosphorylation of IRS-1, a hallmark of insulin resistance (Patel *et al.*, 2016). The phosphorylation of IRS-1 has been reported to play a role in an *in vitro* model of obesity-induced inflammation (Lopez-Vazquez *et al.*, 2017). Here we tested the influence of swertiamarin on the ser307pIRS-1 expression in CCl₄-induced hepatotoxicity. The increased expression of the ser307pIRS-1 in CCl₄-intoxicated rats was notably down-regulated by swertiamarin treatment (200 mg/kg b.w.), suggesting a novel mechanism.

In summary, our findings demonstrate that STM may be an alternative detoxifying medication due to its potential hepatoprotective and anti-inflammatory effects. Although underlying mechanisms have been discussed (Figure 6), further studies need to be explored.

COMPETING INTERESTS

The authors report no conflicts of interest.

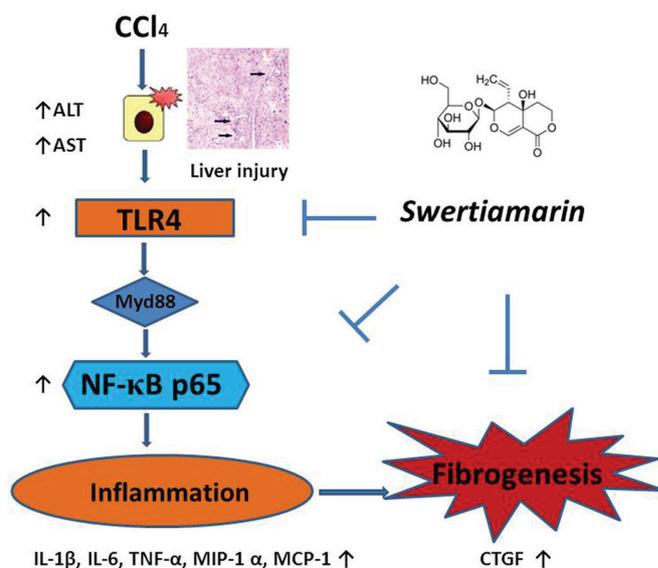


FIGURE 6 - Schematic diagram shows protective signaling of swertiamarin. The \uparrow symbol indicates activation or induction, and \dashv symbol indicates inhibition or blockade.

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