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Studies on the antioxidant and hepatoprotective activities of polysaccharides from *Talinum triangulare*

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ABSTRACT

Ethnophamacological relevancy: The whole plant of *Talinum triangulare* (Family: Portulacaceae) is used in variety of diseases including hepatic ailments in Africa and Taiwan of China.
Aims of the study: The study was aimed to evaluate the antioxidant and hepatoprotective activity of polysaccharides from *T. triangulare* (TTP).
Materials and methods: The TTP was extracted using boiling water, and removed protein by Sevag method. 40%, 60% and 80% ethanol precipitating TTP (40%, 60%, 80% TTP) were gained by the successive addition of absolute ethanol. The antioxidant activities of 40%, 60%, 80% and crude TTP were evaluated using three different models *in vitro*, including reducing power, hydroxyl radicals, superoxide anion. To investigate the hepatoprotective potential, mice were treated with crude polysaccharides (50, 100 and 200 mg/kg, p.o.) for 7 days. Liver injures were induced by CCl_4 (0.1% in arachis oil, 10 mg/kg, i.v.) 1 h after the drug administration on day 7. Mice were sacrificed at 24 h after the CCl_4 injection. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) in serum, and glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA) in liver tissues were measured. Histopathological examinations were carried out to supplement the biochemical results.
Results and conclusions: In vitro assays, TTP showed remarkably different degrees of antioxidant activities in dose-dependent manners. The crude TTP demonstrated a relatively strong antioxidant activity, while the 40% TTP showed the strongest antioxidant activity, and the 60% TTP had the weakest antioxidant ability. *In vivo* assay, pretreatment with TTP had significantly decreased the levels of AST, ALT and MDA against CCl_4 injures, and restored the activities of defense antioxidant substances SOD and GSH towards normalization. These results supported the effect of *T. triangulare* in fork use with scientific evidence.

1. Introduction

Organisms need oxidation for the production of energy to fuel biological processes (Kong et al., 2010). Yet overproduction of oxygen free-radicals, results in oxidative stress, a deleterious process that can be an important mediator of damage to cell structures. It has been found that oxygen free-radicals, or generally known as reactive oxygen species (ROS), are involved in cancer, diabetes, cardiovascular diseases, atherosclerosis, hypertension, ischemia/reperfusion injury, neurodegenerative diseases, inflammation, ageing (Afonso et al., 2007; Valko et al., 2007) and various acute and chronic liver diseases (Bruck et al., 2004). And it is believed that antioxidant agents are helpful for the treatment and prevention of these disorders (Fu et al., 2010). Polysaccharides are among the most widespread and numerous groups of biopolymers. An increasing number of studies have indicated that many polysaccharides extracted from animals, plants, and microorganisms, possess potent antioxidant abilities (Chen et al., 2007).

Talinum triangulare (family Portulacaceae), which originates from tropical Africa, now is widely cultivated as a medicinal and food crop in western Africa, Asia, and South America, especially in Nigeria. In Africa, *T. triangulare* are used intensively and concomitantly with allopathic medicines in the treatment of opportunity diseases by patients or by healthy people to prevent diseases, with the function of increasing stamina and immunostimulant (Agbonon et al., 2009; Fenny et al., 1996). In Taiwan province, the People's Republic of China, *T. triangulare* has been used in the treatment and prevention of hepatic ailments and cancer in folk medicine.

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In a previous study, the aqueous extracts of *T. triangulare* have been proved to possess remarkably antioxidant activity, and the flavonoid content of its extract has been measured (Andarwulan et al., 2010). Yet, the research about *T. triangulare* is far away from enough, for example, little has been done about its polysaccharides, which might possess potent antioxidant and antitumour activities. Our present work aimed to explore the potential of the *in vitro* antioxidant and hepatoprotective activities against CCl_4 induced liver damage.

2. Materials and methods

2.1. Chemicals and plant material

Potassium ferricyanide ($K_3Fe(CN)_6$) was purchased from Tianjin Hengxing Chemical Preparation Corporation (Tianjing, China). 1,10-phenanthroline and pyrogallol were obtained from Sinopharm Chemical Reagent Corporation (Shanghai, China). Assay kits for determination of aminotransferase (AST), alanine aminotransferase (ALT), glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA) were supplied by Nanjing Jiancheng Biotechnology Institute (Nanjing, China). Silymarin was purchased from MADAUS GmbH, Germany.

T. triangulare was purchased from Taiwan, the People's Republic of China, and was authenticated by associate Prof. Zhaoqun Yu, Medical School of Hubei Institute for Nationalities. A voucher specimen (No. 091015) has been deposited in College of Food Science and Technology, Huazhong Agriculture University.

2.2. Preparation of polysaccharides

The dried and powdered whole plant of *T. triangulare* was extracted with distilled water at 90 °C for 2.5 h. The solution was centrifuged at 4000 r/min for 10 min. The supernatant obtained was concentrated under reduced pressure at 50 °C, precipitated upon addition of cold absolute ethanol till the EtOH concentration reached 80% (v/v) and kept overnight. The resulting precipitate was dissolved in distilled water and deproteinized using the Sevag method (Staub, 1965), again precipitated with the addition of cold absolute ethanol till the EtOH concentration reached 80% (v/v) and kept overnight. The resulted precipitates were successively washed with absolute ethanol, acetone and ether, and dried under vacuum at 40 °C, the crude polysaccharides were obtained.

The gained polysaccharides were redissolved in distilled water, absolute ethanol were added till the ethanol concentration reached 40% (v/v) and kept for 24 h, then centrifuged at 4000 r/min for 10 min. The precipitation was gathered and regarded as 40% ethanol precipitating polysaccharides (40% TTP). The absolute ethanol was successively added till the ethanol concentration reached 60% and kept for 24 h, again centrifuged at 4000 r/min for 10 min. The precipitation was gathered and regarded as 60% ethanol precipitating polysaccharides (60% TTP). 80% TTP was obtained by the same way.

2.3. Tested animals

Male Kunming mice (20 ± 2 g, purchased from Hubei Center for Disease Control and Prevention, China) were selected for the study and maintained at a controlled temperature of 25 ± 2 °C and constant humidity (40–70%) under a 12 h light–dark cycle, with free access to diet and water. The animal study was performed according to the international rules considering animal experiments and the internationally accepted ethical principles for laboratory animal use and care.

2.4. In vitro antioxidant activity

2.4.1. Reducing power

The reducing power of 40%, 60%, 80% ethanol precipitating TTP and crude TTP were assessed according to the method described by Chung et al. (2005). Briefly, 2.5 ml of tested polysaccharides sample was mixed with 2.5 ml of 0.2 M phosphate buffer saline (pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$, and then incubated at 50 °C for 20 min. 1 ml of trichloroacetic acid was added to the mixture to stop the reaction, and then the mixture was centrifuged at 3000 r/min for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% $FeC1_3$. The absorbance was measured at 700 nm. The reducing power of the tested sample increased with the absorbance valve.

2.4.2. Hydroxyl radical scavenging activity

The effect of 40%, 60% 80% ethanol precipitating TTP and crude TTP on hydroxyl radical scavenging was determined using the 1,10-phenanthroline method (Jin et al., 1996). The reaction mixture contains 1 ml of 1,10-phenanthroline (7.5 mM), 2 ml of phosphate buffered saline (0.2 M, pH 7.4), 1 ml of $FeSO_4$ (7.5 mM), 1 ml of 0.1% H_2O_2, and 1 ml sample. Distilled water was added in case when total volume was less than 6 ml. After incubation at 37 °C for 1 h, absorbance was measured at 536 nm. Hydroxyl radical scavenging activity was calculated using the follow formula:

$$\text{Hydroxyl radical scavenging activity}\,(\%) = \left[\frac{A_s - A_d}{A_0 - A_d}\right] \times 100$$

where A_s is the absorbance in the presence of both the sample and the H_2O_2, A_d is the absorbance in the presence of the H_2O_2 while the absence of the sample, and A_0 is the absorbance in the absence of both the sample and the H_2O_2.

2.4.3. Superoxide anion scavenging activity

Superoxide anion scavenging activity was evaluated by measuring the inhibition of the auto-oxidation of pyrogallol using a modified method of Marklund and Marklund (1974). 4.5 ml of Tris–HCl buffer (50 mM, pH 8.2) and 4.2 ml distilled water were added into freshly prepared 0.3 ml of 3 mM pyrogallol (dissolved in 10 mM HCl). Pyrogallol was replaced with HCl in the blank control. The absorbance against control at 325 nm was measured at every 0.5 min interval for 4 min. The increment of the absorbance per minute was regarded as the auto-oxidation rate of pyrogallol (A_1). The inhibited auto-oxidation rates of pyrogallol (A_2) were gained by replacing the distilled water with the samples. The ability to scavenging superoxide anion was calculated by the following equation:

$$\text{Superoxide anion scavenging activity}\,(\%) = \left(\frac{A_1 - A_2}{A_1}\right) \times 100$$

2.5. In vivo hepatoprotective activity

2.5.1. CCl_4-induced hepatotoxicity in mice

Mice were divided into six groups ($n = 10$). Group I (normal control) animals were administered a single dose of water (25 ml/kg, p.o.) daily for 7 days and received arachis oil (10 ml/kg, i.p.) on day 7. Group II (CCl_4 control) received water (25 ml/kg, p.o.) once daily for 7 days and received 0.1% CCl_4 in arachis oil (10 ml/kg, i.p.) on day 7. Group 3 (positive control) animals received standard drug silymarin (a drug commonly used in the treatment of liver diseases, 100 mg/kg, p.o.) once daily for 7 days. Groups 4–6 animals were administered 50, 100, 200 mg/kg of polysaccharides once daily for 7 days. Respectively, group 3-6 received 0.1% CCl_4 in arachis oil (10 ml/kg, i.p.) after 1 h of administration of the silymarin and the polysaccharides on day 7.

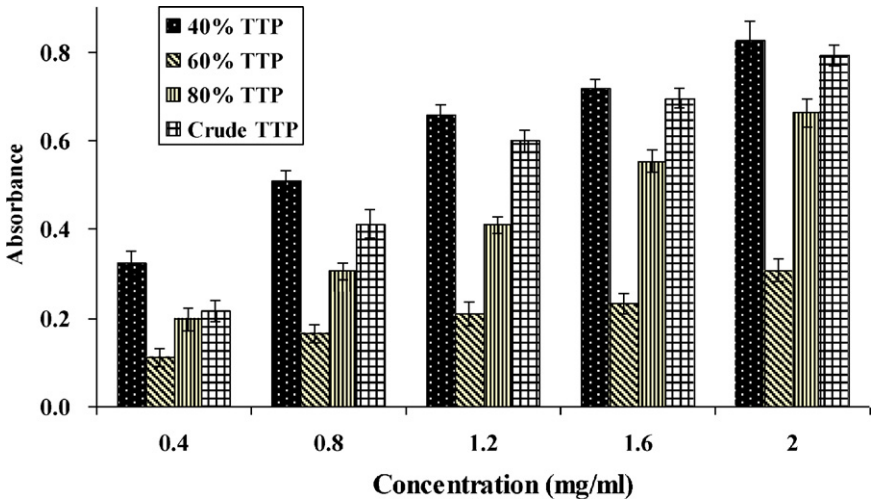

Fig. 1. Reducing power of 40%, 60%, 80% ethanol precipitating TTP and crude TTP. Values are expressed as mean ± standard deviation ($n = 3$).

All animals were sacrificed 24 h after the treatment and blood samples were collected immediately, the livers were removed quickly and dissected to two halves, one for the biochemical studies and the other for the histopathological analysis.

2.5.2. Biochemical assays

The blood samples collected were centrifuged at 3500 rpm for 15 min to obtain serum, which would be used for the assessments of AST and ALT. The liver tissues for the biochemical study were homogenized in 9 volumes of ice NaCl (0.9%), and then the homogenates were centrifuged at 4000 rpm for 10 min to yield a clear supernatant fraction used for GSH, SOD, MDA analysis. All the assessments were conducted by using standard kits supplied by Nanjing Jiancheng Biotechnology Institute (China).

2.5.3. Histopathological studies

Liver tissues were fixed in 10% formalin–saline for 48 h, embedded in paraffin. Sections were prepared by using a rotary microtone, and stained with haematoxylin–eosin dye, observed and recorded.

2.6. Statistical analysis

Data are expresses as mean ± standard deviation (S.D.). Statistical significance of differences between groups was assessed by Student's *t*-test. All calculations were performed in the SAS 9.0. A level of $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Reducing power

The reducing powers of 40%, 60%, 80% ethanol precipitating TTP and crude TTP were obviously dose-dependent (Fig. 1). The absorbance, reflecting the reducing power, increased along with the growing concentration of TTP. 40% TTP possessed the strongest reducing power in every concentration that we have observed. In low concentrations, there was an obvious gap between the absorbance of crude TTP and 40% ethanol precipitating TTP, however, this gap got shorten as the concentration increased.

3.2. Hydroxyl radical scavenging

The effects of 40%, 60%, 80% ethanol precipitating TTP and crude TTP on hydroxyl radical scavenging activity are showed in Fig. 2. Similar to the reducing power, the activities were dose-dependent, and the 40% ethanol precipitating TTP was the strongest part. The scavenging rate of hydroxyl radical reached 89.80% when the con-

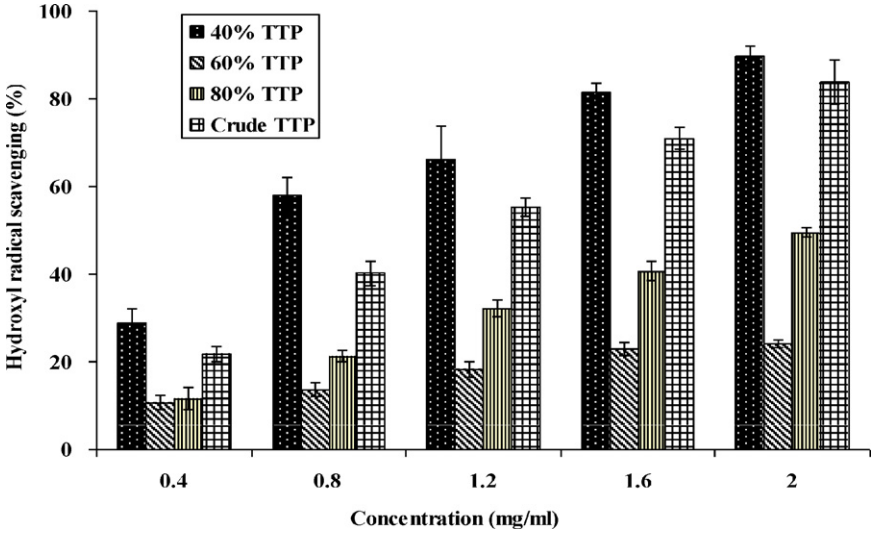

Fig. 2. Hydroxyl radical scavenging activity of 40%, 60%, 80% ethanol precipitating TTP and crude TTP. Values are expressed as mean ± standard deviation ($n = 3$).

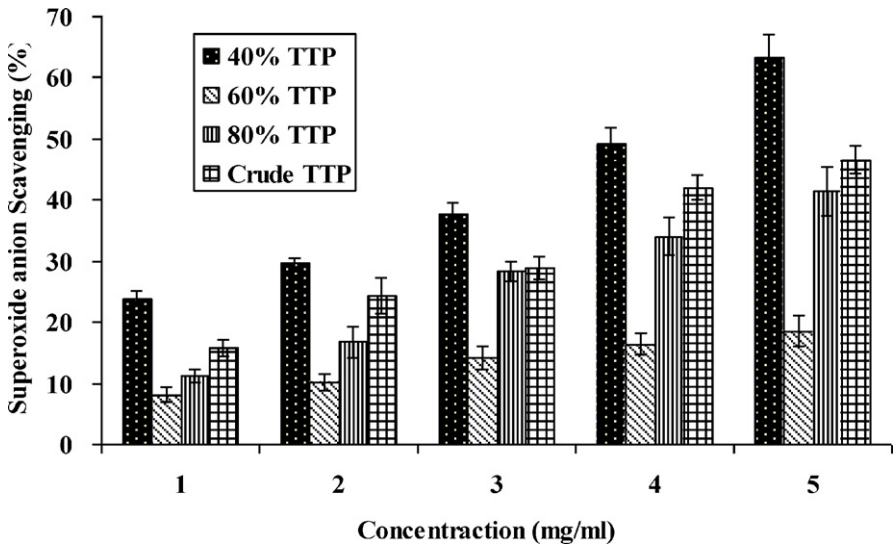

Fig. 3. Superoxide anion radical scavenging activity of 40%, 60%, 80% ethanol precipitating TTP and crude TTP. Values are expressed as mean ± standard deviation ($n = 3$).

centration was 2 mg/ml for 40% ethanol precipitating TTP, while for 60% ethanol precipitating TTP, the scavenging rate was still less than 30% at the same concentration.

3.3. Superoxide anion radical scavenging

The superoxide anion radical scavenging activities of 40%, 60%, 80% ethanol precipitating TTP and crude TTP are showed in Fig. 3, which indicated that the superoxide anion scavenging rate for TTP were concentration-related. But even for the 40% ethanol precipitating TTP, which demonstrated the most potent scavenging activity on superoxide anion radical, the scavenging rate only reached 63.233 ± 3.75% at the concentration of 5 mg/ml.

3.4. CCl_4 induced liver damage in mice

3.4.1. Effects of TTP on the AST and ALT levels in serum

As shown in Table 1, CCl_4 injection produced a significant elevation of both ALT and AST levels in serum compared with normal group ($P < 0.05$), reflecting the tissue damages in liver. Administration of TTP (50, 100 and 200 mg/kg) and silymarin had obviously reversed this elevation towards normal. This reverse effect of TTP was dose-dependent, and the high dose (200 mg/kg) created a therapy comparable to standard drug silymarin at 100 mg/kg.

3.4.2. Effects of TTP on the MDA and GSH levels as well as SOD activities in liver homogenates

As shown in Table 2, compared to normal mice, CCl_4 treatment significantly increased the MDA content and decreased the level/activity of GSH and SOD, suggesting stronger oxidative stress and lipid peroxidation in liver tissue. Pretreatment with (50, 100 and 200 mg/kg) and silymarin had more or less prevented this trend, according to the amount of TTP. When the dose reached 200 mg/kg, the results were nearly as good as silymarin at 100 mg/kg.

3.4.3. Histopathological results

The effect of TTP (50, 100, and 200 mg/kg) and silymarin on liver histopathology of CCl_4 treated mice are presented in Fig. 4. As seen in Fig. 4(A), liver section of normal mice showed distinct hepatic cells with well preserved cytoplasm, prominent nucleus and nucleolus. CCl_4 induced liver damage can be observed directly in Fig. 4(B), where the section showed massive fatty change, necrosis, lymphocyte infiltration, the loss of cellular boundaries, and join together of nucleus. Fig. 4(C) presents the mice section treated with silymarin and CCl_4, Fig. 4(F) shows the section of group treated with high dose TTP (200 mg/kg) and CCl_4, sections of these two groups were nearly comparable to the normal group, with no obvious necrosis was observed. In the 50 mg/kg TTP and CCl_4 treated group, section suggested moderate degree of damage, with some fatty change, necrosis, and lymphocyte infiltration (Fig. 4D). Only very slight lymphocyte infiltration was observed in 100 mg/kg TTP and CCl_4 treated mice (Fig. 4E).

4. Discussion

Oxidative stress is responsible for more than 100 human diseases (Kosecik et al., 2005), and liver diseases remain one of the serious health problems worldwide (Huang et al., 2010). In the present study, we report for the first time the antioxidant and hepatoprotective activities of polysaccharides from *T. triangulare*, which is used in folk medicine to treat and prevent liver ailments and cancers.

Reducing power, reflecting the electron donation capacity, is one of the most important indicators of antioxidant activity of bioactive compounds (Arabshahi-Delouee and Urooj, 2007). By donating electrons, antioxidant substances are able to block radical chain reaction by converting reactive oxygen species to more stable products. In our experiment, when TTP is added to the reaction mixture, it can reduce the Fe^{3+}/ferricyanide complex to the ferrous form, which can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Ak and Gulcin, 2008).

Hydroxyl radicals have the strongest chemical activity among various reactive oxygen species, they can damage a wide range of essential biomolecules such as amino acid, protein, and DNA (Halliwell and Gutteridge, 1990). However, there is no specific enzyme to defense against hydroxyl radicals in human body. Therefore, it would be of great significance to discover some compounds with good hydroxyl radical scavenging activity for the oxidative stress induced diseases (Zhou et al., 2010). Studies have shown that polysaccharides with hydroxyl radicals scavenging ability have the same structure feature that have one or more alcohol or hydroxyl groups, and the scavenging capacity is related to the amount of these hydroxyl active groups (Yang et al., 2010).

Table 1
Effects of TTP administration on the levels of AST and ALT in serum in the liver damaged mice induced by CCl_4.

Group	Treatment	AST (U/L)	ALT (U/L)
I	Control	56.29 ± 8.65	118.49 ± 6.12
II	CCl_4 (10 ml/kg, i.p.)	134.50 ± 10.19[a]	220.33 ± 15.32[a]
III	CCl_4 + silymarin (100 mg/kg, p.o.)	67.70 ± 7.09[b]	128.47 ± 8.20[b]
IV	CCl_4 + TTP (50 mg/kg, p.o.)	111.35 ± 9.19[b]	155.83 ± 13.53[b]
V	CCl_4 + TTP (100 mg/kg, p.o.)	86.32 ± 9.13[b]	135.13 ± 17.42[b]
VI	CCl_4 + TTP (200 mg/kg, p.o.)	72.63 ± 6.87[b]	121.39 ± 8.02[b]

Values are expressed as mean ± standard deviation (n = 10).
[a] Significance level: $P < 0.05$, compared to normal group.
[b] Significance level: $P < 0.05$, compared to CCl_4 group.

Table 2
Effects of TTP administration on MDA and GSH levels as well as SOD activities in liver homogenate in the liver damaged mice induced by CCl_4.

Group	Treatment	MDA (nmol/mg protein)	SOD (U/mg protein)	GSH (nmol/mg protein)
I	Control	0.22 ± 0.06	110.46 ± 11.32	128.36 ± 20.05
II	CCl_4 (10 ml/kg, i.p.)	0.57 ± 0.07[a]	77.35 ± 7.21[a]	80.24 ± 11.63[a]
III	CCl_4 + silymarin (100 mg/kg, p.o.)	0.27 ± 0.04[b]	104.83 ± 9.81[b]	125.38 ± 14.92[b]
IV	CCl_4 + TTP (50 mg/kg, p.o.)	0.47 ± 0.05[b]	89.48 ± 9.90[b]	103.12 ± 18.71[b]
V	CCl_4 + TTP (100 mg/kg, p.o.)	0.37 ± 0.06[b]	96.43 ± 9.68[b]	110.58 ± 17.53[b]
VI	CCl_4 + TTP (200 mg/kg, p.o.)	0.29 ± 0.07[b]	102.45 ± 8.55[b]	121.76 ± 16.48[b]

Values are expressed as mean ± standard deviation (n = 10).
[a] Significance level: $P < 0.05$, compared to normal group.
[b] Significance level: $P < 0.05$, compared to CCl_4 group.

Although it is a relatively weak oxidant, super anion radical is one of the most common free radicals generated *in vivo*, and is one of the precursors of singlet oxygen and hydroxyl radical, which will cause tissue damages. Excessive production of superoxide anion radical has been regarded as the beginning of ROS accumulation in cells, resulting in redox imbalance and related harmful physiological consequences (Li et al., 2010). It is postulated that the superoxide anion radical scavenging ability of polysaccha-

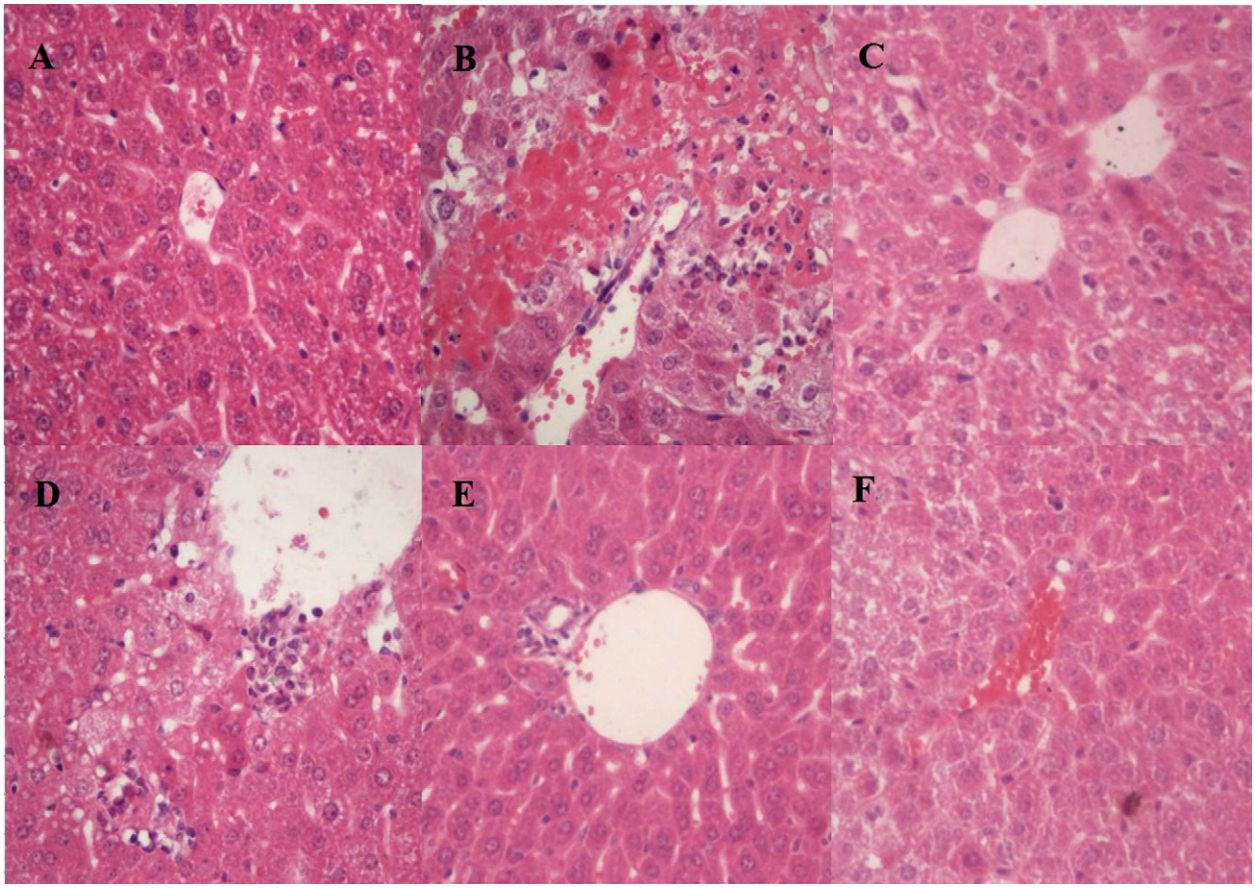

Fig. 4. Effect of TTP (50, 100, and 200 mg/kg) and silymarin on liver histopathology of CCl_4 treated mice. (A) Liver section of normal group; (B) liver section of CCl_4 control; (C) liver section of silymarin (100 mg/kg) + CCl_4 group; (D) liver section of TTP (50 mg/kg) + CCl_4 group; (E) liver section of TTP (100 mg/kg) + CCl_4 group; (F) liver section of TTP(200 mg/kg) + CCl_4 group.

rides is related to the weak dissociation energy of its O–H bond, which would make it easier to donate hydrogen to superoxide anion (Chang et al., 2010).

CCl_4 induced hepatotoxicity is the common experimental model for the hepatoprotective drug screening. CCl_4 is metabolized to the trichloromethyl radical ($^{\bullet}CCl_3$) and proxy trichloromethyl radical ($^{\bullet}OOCCl_3$) by cytochrome P450 2E1 enzyme (Jia et al., 2011). These radicals bind covalently to the macromolecules and cause peroxidative degradation of cellular lipid membrane, which will cause the loss of integrity of cell membranes, and the necrosis of hepatocytes (Ranawat et al., 2010). Liver damage can be assessed by biochemical studies. AST and ALT levels are most frequently used in the diagnosis and management of liver diseases. AST and ALT are present in high concentration in hepatocytes, while they leak into the circulation when hepatocytes or their membranes are damaged (Kew, 2000). MDA level in liver tissue is used to reflect the extent of lipid peroxidation in hepatocytes, as MDA is one of the end-products of polyunsaturated fatty acid peroxidation (Esterbauer et al., 1991). Hepatic GSH plays a crucial role in scavenging ROS and maintaining enzymatic antioxidant as an important non-enzymatic antioxidant. In the CCl_4 treated mice GSH plays a key role in eliminating the reactive toxic metabolites of CCl_4 (Ou et al., 2010). SOD is one of the major antioxidant enzymes responsible for the defense against potentially free radicals that cause oxidative stress, but it is highly sensitive to and easily inactivated by lipid peroxide and ROS.

As what was observed in our experiment, the injection of CCl_4 led to the elevation of AST and ALT levels in serum and MDA level in liver tissue, while the decreasing of GHS content and SOD activity, all of which reflecting the liver damage. Pretreatment with TTP had reversed these trends towards normalizations, reflecting that TTP possesses potent hepatoprotective activity *in vivo*, which might attribute to its strong antioxidant activity. Histopathological observation of the liver tissue had directly supported this conclusion.

One major feature of our experiment is that we used 40%, 60% and 80% ethanol precipitation fragments as well as crude TTP in the *in vitro* assays. The results demonstrate that 40% TTP has the strongest reducing power, hydroxyl radical, and superoxide anion scavenging activities. Hence, more researches are needed on 40% TTP to make it a novel medicine.

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