



Antioxidant and hepatoprotective effects of *Schisandra chinensis* pollen extract on CCl₄-induced acute liver damage in mice

Ni Cheng^{a,b}, Naiyan Ren^b, Hui Gao^b, Xingsheng Lei^c, Jianbin Zheng^{a,*}, Wei Cao^{b,c,*}

^a Institute of Analytical Science, Shaanxi Provincial Key Lab of Electroanalytical Chemistry, Northwest University, 229 North TaiBai Road, Xi'an City 710069, China

^b Department of Food Science and Engineering, Northwest University, 229 North TaiBai Road, Xi'an City 710069, China

^c Bee Product Research Center of Shaanxi Province, Xi'an City 710065, China

ARTICLE INFO

Article history:

Received 31 August 2012

Accepted 16 November 2012

Available online 28 November 2012

Keywords:

Schisandra chinensis pollen extract (SCPE)

Antioxidant

Hepatoprotective effects

CCl₄

ABSTRACT

The aim of the present study was to investigate the antioxidant and hepatoprotective effects of *Schisandra chinensis* pollen extract (SCPE) on CCl₄-induced acute liver damage in mice. Total phenolic content, total flavonoid content, individual phenolic compounds and antioxidant activities (1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, chelating activity, and reducing power assay) were determined. In vivo study, SCPE (10, 20 and 40 g/kg) administered daily orally for 42 days prior to CCl₄-intoxicated. Our results showed that SCPE had high total phenolic content (53.74 ± 1.21 mg GAE/g), total flavonoid content (38.29 ± 0.91 mg Rutin/g), quercetin and hesperetin may be the major contributor to strong antioxidant activities. Moreover, SCPE significantly prevented the increase in serum ALT and AST level in acute liver damage induced by CCl₄, decreased the extent of malondialdehyde (MDA) formation in liver and elevated the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in liver. The results indicated that SCPE has strong antioxidant activities and significant protective effect against acute hepatotoxicity induced by CCl₄, and have been supported by the evaluation of liver histopathology in mice. The hepatoprotective effect may be related to its free radical scavenging effect, increasing antioxidant activity and inhibiting lipid peroxidation.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Carbon tetrachloride (CCl₄) is one of the oldest and most widely used toxins for experimental induction of liver injury in laboratory animals (Brautbar and Williams, 2002). It is generally accepted that the hepatotoxicity of CCl₄ is the result of reductive dehalogenation, which is catalyzed by hepatic cytochrome P-450, and which forms unstable trichloromethyl (CCl₃) and trichloromethyl peroxy (CCl₃O₂) radicals (Brattin et al., 1985). Both trichloromethyl and its peroxy radical are capable of binding to proteins or lipids, or of abstracting a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation and liver damage (Recknagel et al., 1989). Lots of studies indicate that natural substances from edible and medicinal plants exhibited strong antioxidant activity that could act against CCl₄-induced liver damage (Yeh et al., 2012; Yang et al., 2010; Desai et al., 2012; Sreelatha et al., 2008), because they contain lots of free radical scavenger such as phenolic acids and flavonoid compounds.

* Corresponding authors at: Department of Food Science and Engineering, Northwest University, 229 North TaiBai Road, Xi'an City 710069, China. Tel.: +86 29 88303448; fax: +86 29 88302213.

E-mail addresses: jbzheng@nwu.edu.cn (J. Zheng), caowei@nwu.edu.cn (W. Cao).

Bee pollen is the male seed of a flower blossom, collected by honey bees by adding sugars from nectar and secretion of honey bees to bind the grains together. It has been used as a health food supplement, even used as medicine for many years (Linskens and Jorde, 1997) due to its abundant nutrient properties including sugars, proteins, lipids, vitamins, carbohydrates and phenolic compounds (Campos et al., 2003). Phenolic compounds are one of the most critical ingredients related antioxidant activity in pollen. Usually, it contains vanillic acid, protocatechuic acid, gallic acid, *p*-coumaric acid, hesperidin, rutin, kaempferol, apigenin, luteolin, quercetin, and isorhamnetin (Chu et al., 2007; Bonvehí et al., 2001). This composition tends to be species-specific and be related to the therapeutic properties (antibiotic, antineoplastic, anti-diarrhoeic and antioxidant) of pollen (Märghitaş et al., 2009; Campos et al., 1997; Almaraz-Abarca et al., 2004). More specifically, the ingestion of bee pollen by rats has also been shown to decrease the level of the lipid oxidation products and increase the activities of antioxidant enzymes (Dudov and Starodub, 1994; Šarić et al., 2009).

Schisandra chinensis is widely distributed in China, and has been used as Chinese remedy for liver damage for several centuries. Many studies have reported that *S. chinensis* contains dibenzocyclooctadiene lignans, such as Schizarin A, B, C, D and E, and exhibits the function of hepatoprotective, antioxidant,

anti-lipid peroxidative, anticancer and anti-HIV (Kuo et al., 2001; Chen et al., 2002, 1996; Yang et al., 1992; Zhang et al., 2009). Those studies mainly focus on the stem, root and seed of the vegetable. The bee pollen of *S. chinensis*, accounting for about 15% of total pollen production in China, has been sold as functional food in China for several decades. However, very little if any research has been done on the antioxidant activities of the pollen, especially undertaking on hepatoprotective effect on CCl₄-induced acute liver damage in rats.

The objectives of our study were: (1) to determine the total phenolic content (TPC), total flavonoid content (TFC) and individual phenolic compounds of *S. chinensis* pollen extract (SCPE); (2) and determine its free radical scavenging activity in vitro, reducing power and Ferrous ion-chelating activity; (3) to investigate the protective effects of SCPE on CCl₄-induced hepatic damage, including the effects of SCPE on the biochemical determinations of the levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and the levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) in liver homogenate combined with hepatic histopathological observations in vivo.

2. Materials and methods

2.1. Chemicals and reagents

The diagnostic kits for AST, ALT, MDA, SOD, GSH-Px and protein were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Folin-Ciocalteu's phenol reagent, DPPH (1,1-diphenyl-2-picrylhydrazyl), ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4'-yl-disulfonic acid monosodium salt), protocatechuic acid, vanillic acid, *p*-coumaric acid, gallic acid, rutin, resveratrol, quercetin, hesperetin, kaempferol, galangin were purchased from Sigma-Aldrich (Steinheim, Germany). CCl₄, peanut oil, iron (II) sulfate 7-hydrate (FeSO₄·7H₂O), sodium carbonate, and ethanol were purchased from Beijing Chemical Co. (Beijing, China). HPLC grade methanol was purchased from Merck (Darmstadt, Germany). HPLC grade water was purified by Milli-Qsystem (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade and were purchased from Xi'an Chemical Co. (Xi'an, China).

2.2. Preparation of SCPE

Bee pollen of *S. chinensis* was provided by Shaanxi Bee Master Co., Ltd. (Xi'an, China). Its floral origin was identified by color and light microscope examination by palynological analysis at 1000× magnification using a bright-field microscope (Olympus, Tokyo). Pollen type was identified by comparison with pollen morphology of *S. chinensis* proposed by Ai et al. (2007) and Sun (2000). The sample was extracted twice with 15 times of 70% (v/v) ethanol for 2 h under reflux followed by centrifugation at 4800 rpm for 10 min to collect supernatant. The combined supernatant was filtered with a funnel and concentrated between 40 and 50 °C and with a degree of vacuum at 0.08 MPa. Then it was freeze-dried to powder. The extraction efficiency was calculated according to the following equation: extraction efficiency (%) = (m_0/m) × 100, where m_0 was the weight of freeze dried powder and m was the weight of pollen.

SCPE was dissolved in methanol for all determination in vitro, and dissolved in distilled water for animal experiment.

2.3. Total phenolic content (TPC), total flavonoid content (TFC) and HPLC analysis

TPC was determined using a modified version of the Folin-Ciocalteu method (Zhou et al., 2012). Nought point four milliliter of SCPE solution (2 mg/mL) was added to 1.0 mL of Folin-Ciocalteu reagent and mixture was kept at room temperature for 5 min. Five milliliter of sodium carbonate (1 M) was added to the mixture and the whole mixed gently. The total volume of the mixture was adjusted to 10 mL with distilled water. After the mixture was kept at room temperature for 1 h, the absorbance was read at 760 nm with a 751 UV-visible spectrophotometer (Shanghai Eastsen Analytical Instrument Co., Ltd., China). The standard calibration (0.02–0.12 mg/mL) curve was plotted using gallic acid. The TPC was expressed as the gallic acid equivalents per gram SCPE (mg GAE/g).

TFC was determined according to the method proposed by Jia et al. (1999) with a slightly modification. One milliliter of SCPE solution (2 mg/mL) was placed in a 10 mL volumetric flask and 0.4 mL of 5% sodium nitrite solution was added. Nought point four milliliter of 10% aluminum nitrate was added 6 min later. After 6 min, 4 mL of 4% sodium hydroxide was added and the total was made up to 10 mL with methanol. The solution was mixed well again and the absorbance was measured

against a blank at 510 nm 15 min later. Rutin was used as the standard for a calibration curve. The TFC was expressed as the rutin equivalents per gram SCPE (mg Rutin/g).

Individual phenolic compounds were analyzed according the method reported by Liang et al. (2009) with slight modifications. HPLC was performed with an Agilent 1100 liquid chromatography system (Agilent Technologies Deutschan, Waldbronn, Germany), equipped with a vacuum degasser, a quaternary solvent delivery pump, a manual chromatographic valve, a thermostated column compartment, a diode-array detector (Agilent, Palo Alto, CA, USA) and a HP 1049A programmable electrochemical detector (HP, USA) was used. The samples were dissolved in methanol and filtered through a 0.45 μm membrane filter. The column is a Zorbax SB-C18 column (150 × 4.6 mm, 5.0 μm). The mobile phase adopted was methanol (B) and 2 % aqueous acetic acid (A) (v/v) using a linear gradient elution of 2–8% B at 0–6 min, 8–15% B at 6–10 min, 15–35% B at 10–15 min, 35–55% B at 15–20 min, 55–65% B at 20–25 min, 65–70% B at 25–30 min, 70–70% B at 30–35 min. The flow-rate was kept at 1.0 mL/min at all times. The column was operated at room temperature. The injection volume was 10.0 μL. The diode-array detector was performed at 360 nm and the electrochemical detector was set at 0.8 V in the oxidative mode.

2.4. Analysis of antioxidant activities in vitro

2.4.1. DPPH radical scavenging activity

Scavenging activity on DPPH free radical by SCPE was assessed according to the method reported by Wang et al. (2012) with slight modifications. Briefly, different volumes of the sample (12.5 mg/mL) were placed in a cuvette with 4.0 mL of 0.1 mM methanolic solution of DPPH radical added. The total volume of mixture was adjusted to 10 mL with distilled water. Then, the mixture was shaken evenly and allowed to stand at room temperature in the dark for 30 min. Thereafter, the absorbance of the assay mixture was measured at 517 nm against methanol blank using a spectrophotometer. DPPH radical scavenging capacity was expressed as the percentage inhibition of DPPH radical. The percentage inhibition of DPPH radical by SCPE was calculated from the absorbance value according to the following equation:

$$\text{Inhibition of DPPH radical(\%)} = (A_0 - A_t)/A_0 \times 100$$

where A_0 was the absorbance of control (blank, without sample) and A_t was the absorbance in the presence of sample.

2.4.2. Reducing power assay

The reducing power of SCPE was determined by the method of Estevinho et al. (2008) with a slight modification. Different volumes of sample (10 mg/mL) were adjusted to 1 mL with distilled water. Two point five milliliter of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide were added. The mixtures were incubated in water bath for 20 min at 50 °C. At the end of the incubation, 1 mL of 10% trichloroacetic acid was added into the mixtures. Centrifugation at 3000 rpm for 10 min to collect supernatant was implemented if needed. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture correlates with greater reducing power.

2.4.3. Ferrous ion-chelating activity

The ferrous ion-chelating activity of SCPE was investigated according to the method of Nandita and Rajini (2004) by measuring the absorbance of ferrozine-Fe²⁺ complex at 562 nm. Briefly, the reaction mixture, containing 30 μL of sample (10 mg/mL), iron vitriol (1 mM) 50 μL and ferrozine (1 mM) 20 μL, was adjusted to a total volume of 1 mL with methanol, shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against the blank. The standard calibration (0.05–0.25 mg/mL) curve was plotted using Na₂EDTA. Ferrous ion-chelating activity was expressed as the Na₂EDTA equivalents (mg Na₂EDTA/g SCPE).

2.5. Animals experiment

2.5.1. Animals

Male Kunming mice (weighing 18–22 g), obtained from the Experimental Animal Center of Xi'an Jiaotong University, were used. They were allowed free access to standard dry pellet diet, which was made by Xi'an QinLe Feed Co., Ltd., and water ad libitum. All animals were grouped and housed in polyacrylic cages (29 × 18 × 16 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C, relative humidity 50 ± 10%) with dark and light cycle (14/10 h). The mice were acclimatized to laboratory condition for 5 days before commencement of experiment. All the experiments were performed in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China.

2.5.2. CCl₄-induced hepatotoxicity model

Mice were randomly divided into five groups of 12 animals each. In the control group and CCl₄-intoxicated group, animals were given a single dose of distilled water (0.2 mL/10 g body weight) orally daily using a gavage. In the test groups, animals were given 10, 20, and 40 g of SCPE per kilogram body weight orally daily using a gavage. All administrations were conducted for 6 weeks. On the 42nd day, all mice except those in the control group were given simultaneously a CCl₄/peanut oil mixture (0.2:100, intraperitoneally, 0.1 mL/10 g body weight) 2 h after the last administration, while the control group received peanut oil alone. Then all the animals were fasted for 16 h and were subsequently tested for the following analysis.

2.5.3. Assessment of liver function

After blood collection, serum was separated by centrifugation at 3000 rpm at room temperature for 20 min. The serum ALT and AST values were measured with commercially available diagnostic kits.

2.5.4. Determination of MDA, SOD and GSH-Px activity

Liver was excised immediately after the animals were sacrificed. The liver, except a portion of the left lobe to be used for histopathological sections, was frozen quickly and stored at -80°C . Prior to determinations, thawed tissue samples were homogenized in 9 volumes of ice cold 50 mM phosphate buffer (pH 7.4), centrifuged at 2500 rpm for 20 min at 4°C . The supernatant was used for determination of MDA, SOD, GSH-Px and protein concentration using commercially available diagnostic kits. The levels of MDA, SOD and GSH-Px were normalized with protein.

2.5.5. Histopathological examinations

A portion of the left lobe of the liver was preserved in 10% neutral formalin solution for at least 24 h, processed and paraffin embedded as per the standard protocol. Sections of 5 μm in thickness were cut, deparaffinized, dehydrated, and stained with haematoxylin and eosin (H&E) for the estimation of hepatocyte necrosis and vacuolization. Morphological changes were observed including cell gross necrosis, sinusoidal congestion, fatty change, ballooning degeneration, inflammatory infiltration.

2.6. Statistical analysis

All the tests were performed in triplicate. Data analysis as carried out using SAS software, version 8.1 (SAS Institute, Cary, NC, USA). Statistically significant differences between the samples were evaluated by the Tukey's test. Differences at $P < 0.05$ were considered to be significant.

3. Results and discussion

3.1. Total phenolic content (TPC), total flavonoid content (TFC) and HPLC analysis

Phenolic compounds may contribute directly to antioxidant action, because of their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Negri et al., 2011). Therefore, TPC and TFC of the SCPE were investigated in our study. TPC of SCPE was 53.74 ± 1.21 mg GAE/g, and TPC of *S. chinensis* pollen was 25.41 ± 1.38 mg GAE per gram pollen (extraction efficiency was $47.28 \pm 0.51\%$). It was higher than majority of pollens. In the previous studies, Negri et al. (2011) found the TPC of seven pollen samples from Southeast Brazil ranged from 1.6 to 2.3 mg GAE per gram pollen. TPC of twelve honeybee-collected pollens in Romania were estimated according to Folin–Ciocalteu

Table 1
Individual phenolic compounds (mg/kg) of SCPE.

Compound	Concentration
Gallic acid	5.78 ± 0.01
Protocatechuic acid	4.55 ± 0.07
Vanillic acid	14.92 ± 0.03
<i>p</i> -Coumaric acid	10.86 ± 0.03
Resveratrol	177.15 ± 0.17
Quercetin	719.93 ± 0.13
Hesperetin	285.35 ± 0.20
Kaempferol	95.47 ± 0.01
Galangin	365.41 ± 0.08

Results presented in the table were expressed as the mean values \pm standard deviation (SD) for 3 replications.

method, ranging from 4.4 to 16.4 mg GAE per gram pollen (Märghitaş et al., 2009). TFC of SCPE was 38.29 ± 0.91 mg Rutin/g, which was higher than rape pollen from different provinces of China (Cheng et al., 2011).

Individual phenolic compounds of SCPE were determined by HPLC with diode-array detector and electrochemical detector. The results of quantitative determination were showed in Table 1. Phenolic acids (gallic acid, protocatechuic acid, vanillic acid, *p*-coumaric acid,) and flavonoid compounds (resveratrol, quercetin, hesperetin, kaempferol, galangin) were presented in SCPE. The content of quercetin (365.41 mg/kg) is the highest among identified phenolic compounds, followed by galangin, hesperetin, resveratrol. The total quantified phenolic acids were 36.11 mg/kg, whereas total quantified flavonoid compound could reach to 1643.31 mg/kg that was about 45 times than the former. The result demonstrated that the predominant phenolic antioxidants in pollen were flavonoid compound, which was in agreement with previous investigation (Negri et al., 2011; Bonvehí et al., 2001). However, only 9 individual phenolic compounds were identified and quantified in SCPE in our study. It should not be ignored that some other compounds, which were response to electrochemical detector, were not identified under the experimental conditions. Those compounds might make a great contribution to antioxidant activities of SCPE. Among identified and quantified phenolic compounds, owing to super antioxidant activities, quercetin and hesperetin had been investigated and proved to possess hepatoprotective effects in mice (Tirkey et al., 2005; Gilani et al., 1997; Lee et al., 2010). In short, SCPE possessed abundant phenolic compounds,

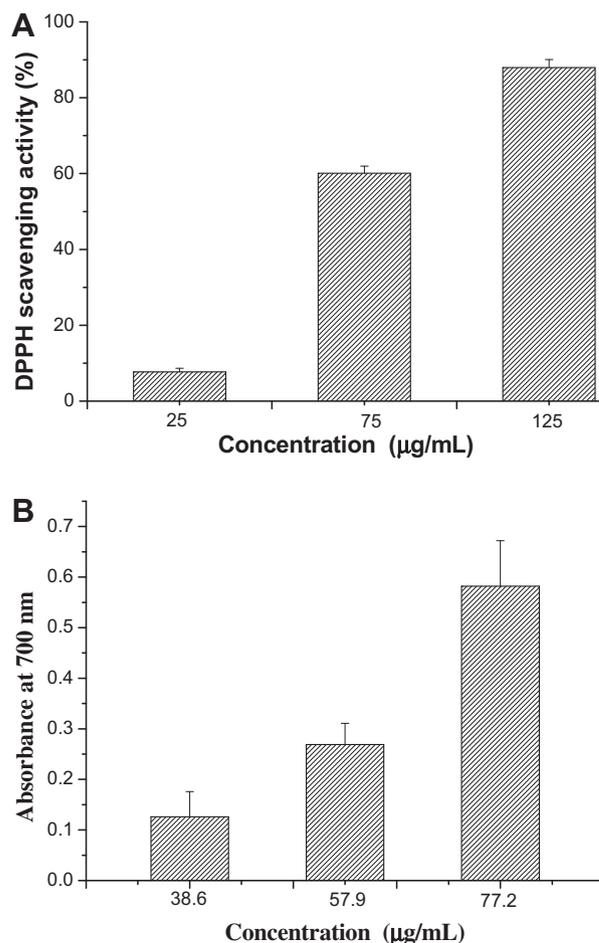


Fig. 1. Antioxidant activities of SCPE in DPPH scavenging activity (A) and reducing power (B).

which made SCPE contain high antioxidant activities and might protect liver from CCl₄ damage.

3.2. Analysis of antioxidant activities in vitro

In order to analysis the antioxidant activities of SCPE in vitro, we monitored three items and the results were as follows.

3.2.1. DPPH radical scavenging activity

DPPH was a widely used stable free radical to evaluate antioxidant activities of bioactive compounds and food extracts. In this study, the DPPH radical scavenging activities of SCPE increased in a concentration-dependent manner (Fig. 1A). When the concentration of sample ranged from 25 to 125 µg/mL, the percentage DPPH scavenging activity ranged from 7.68% to 87.94%. SCPE exhibited a dose dependent scavenging of DPPH radical in a measurement concentration range. The high radical scavenging activity should attribute to antiradical of phenolic compounds (Soares et al., 1997; Yildirim et al., 2001; Jadeja et al., 2009).

3.2.2. Reducing power assay

The presence of reductants in sample would result in the reduction of the ferric ion/ferricyanide complex to its ferrous form. The amount of ferrous ion complex could then be monitored by measuring the formation of Perli's Prussian blue at 700 nm. Ferric ion-reduction was often used as an indicator for electro-donating activity of antioxidant, which was an important mechanism of phenolic antioxidant action (Yildirim et al., 2001). In this study, it was not surprising to see a trend of increasing reducing power following the increasing sample concentration (Fig. 1B). Previous studies indicated that there was a high correlation between the reducing power and phenolic compounds (Zhou et al., 2012; Yildirim et al., 2001). Therefore, strong reducing ability was observed in phenolic compound-enriched SCPE.

3.2.3. Ferrous ion-chelating activity

Ferrous ions chelation might render important antioxidative effects by retarding metal-catalyzed oxidation. The ability of SCPE to chelate ferrous was 23.24 ± 0.79 mg Na₂EDTA per gram extract, indicating SCPE possessed strong chelating activity. Generally, flavonoid compound, such as quercetin and rutin, were significantly

effective inhibitors of iron ion-dependent lipid peroxidation systems due to chelating iron ions with the formation of inert iron complexes unable to initiate lipid peroxidation (Sun et al., 2008; Afanas'ev et al., 1989). SCPE possessed abundant flavonoid (38.29 ± 0.91 mg Rutin/g), therefore, it could show strong chelating activity and devote to antioxidant powerfully.

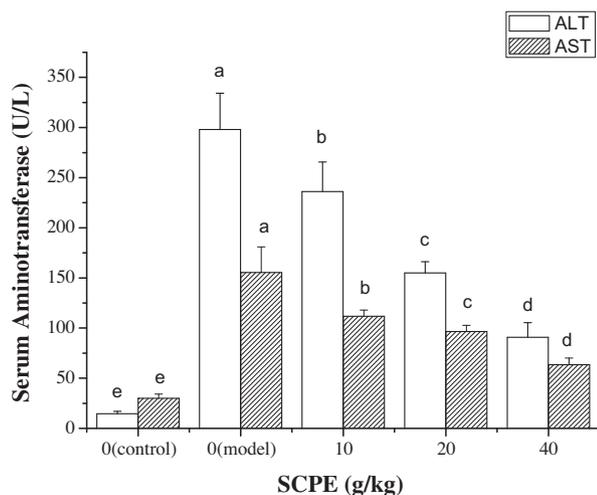


Fig. 2. Effects of SCPE on serum ALT and AST activity. Different lower case letters correspond to significant differences at $p < 0.05$. Animals were given orally SCPE (0, 10, 20, 40 g/kg) once daily for 42 days prior to the administration of CCl₄ (0.2%, ip) except 0 (control), which accepted the administration of peanut oil.

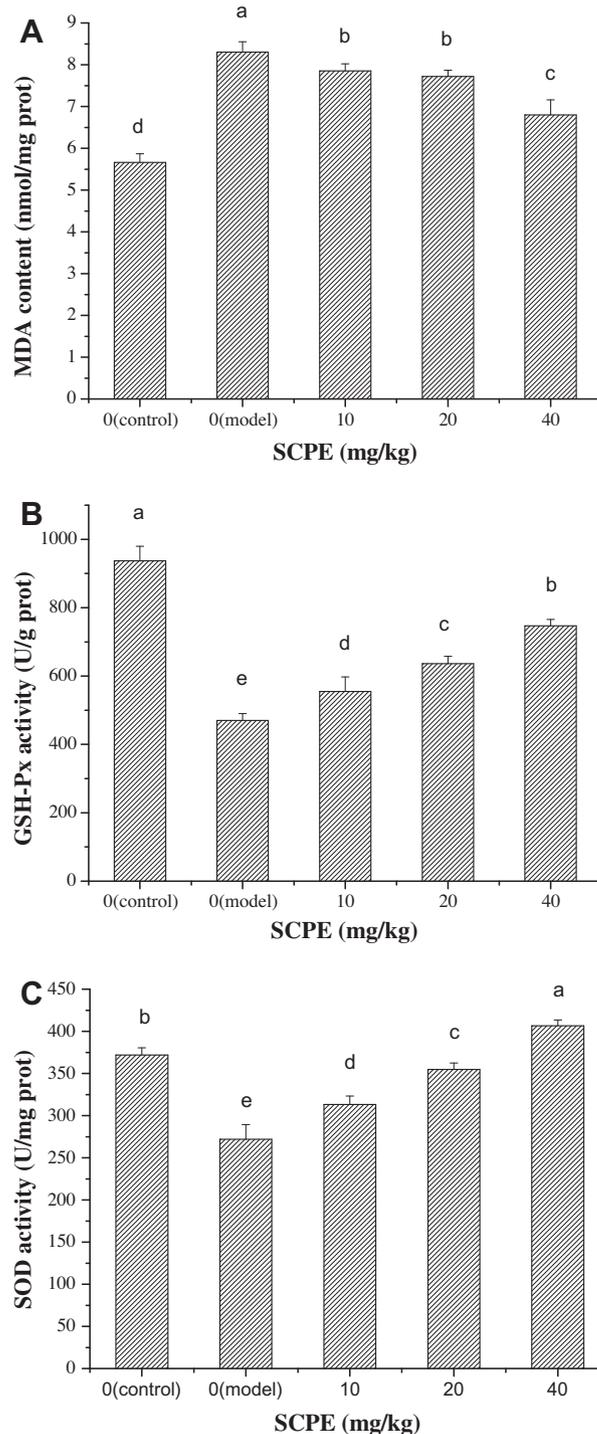


Fig. 3. Effects of SCPE on the hepatic GSH-Px activity (A), MDA content (B) and SOD activity (C). Different lower case letters correspond to significant differences at $p < 0.05$. Animals were given orally SCPE (0, 10, 20, 40 g/kg) once daily for 42 days prior to the administration of CCl₄ (0.2%, ip) except 0 (control), which accepted the administration of peanut oil.

3.3. Animals experiment

The results of hepatoprotective effect of SCPE on the serum ALT and AST activities were shown in Fig. 2. In the model group (CCl₄-intoxicated group), serum ALT and AST activities were 298.15 and 155.48 U/L respectively, whereas the values of control group were only 14.47 and 30.08 U/L, respectively. Therefore, a significant increase in the activities of serum ALT and AST was observed when liver was exposure to CCl₄ ($P < 0.05$). Administration with 10, 20, 40 g of SCPE for 42 days significantly reduced 20.81%, 47.99%, 69.80% of the activities of serum ALT, and 9.01%, 38.06%, 59.35% of the activities of serum AST as compared to the model group ($P < 0.05$).

In this study, the product of membrane lipid peroxidation (MDA) was also observed. Its content increased about 46.64% in CCl₄-intoxicated mice compared with that of control group (Fig. 3A). Different dose of SCPE showed significant suppression on MDA formation ($P < 0.05$). The content of MDA in 40 g of SCPE decreased 18.07% than CCl₄ model group. Significant lower activities of SOD and GSH-Px were observed in CCl₄ model group mice liver as compared to the control group (Fig. 3B and C). GSH-Px activity of CCl₄ model group was only 470.2 U/g prot, which was

reduced by half compared to control group (937.14 U/g prot). Different dose of SCPE groups showed a significant increase ($P < 0.05$) than model group in a dose-dependent manner. Although GSH-Px activity of the largest dose (40 g/kg) was lower than control group significantly ($P < 0.05$), SCPE presented increasing GSH-Px activity in impaired liver. SOD activity (406.61 U/mg prot) of administrated with 40 g/kg prior to CCl₄-intoxicated was significantly higher than CCl₄ model group (272.01 U/mg prot) ($P < 0.05$) and even higher than control group (372.04 U/mg prot) ($P < 0.05$).

The protective effects exerted by SCPE against CCl₄-induced hepatotoxicity were further confirmed by conventional histological assessment (Fig. 4). The histology of the liver sections of control group showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus, visible central veins and thin sinusoids (Fig. 4A). The stained sections of CCl₄ model group revealed extensive liver injuries characterized by moderate to severe hepatocellular hydropic degeneration and necrosis around the central vein, inflammatory cell infiltration, ballooning degeneration and dilated sinusoidal spaces (Fig. 4B). However, CCl₄-intoxicated mice pretreated with 10 g/kg of SCPE showed moderate hypertrophy of hepatocytes with relatively intact central vein, shrinking sinusoidal and reducing inflammatory cell (Fig. 4C). The histopa-

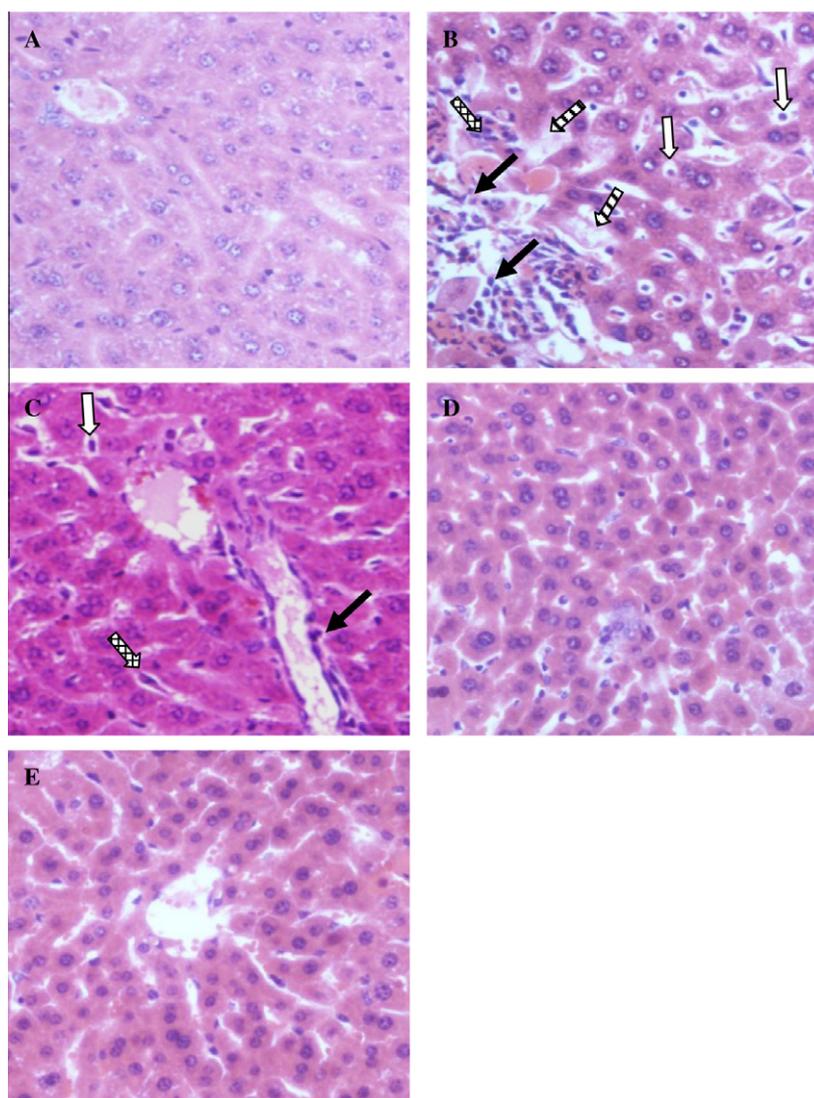


Fig. 4. Effects of SCPE on hepatic morphological analysis ($\times 400$ H&E): control group (A), CCl₄-model group (B), SCPE (10 g/kg) and CCl₄ group (C), SCPE (20 g/kg) and CCl₄ group (D), SCPE (40 g/kg) and CCl₄ group (E). (—▶, inflammatory infiltration; —▶, ballooning degeneration; —▶, hepatocellular necrosis; —▶, dilated sinusoidal spaces).

thological hepatic lesions were markedly ameliorated by pretreatment with 20, 40 g/kg SCPE (Fig. 4D and E).

In the animals experiment, the function of SCPE to protect against CCl₄-induced hepatotoxicity was investigated. CCl₄ hepatotoxicity derives from its' metabolites (highly reactive trichloromethyl free radicals). This radical could react with oxygen to form the trichloromethylperoxy radical CCl₃OO, a highly reactive species that initiated the chain reaction of lipid peroxidation, and attacked and destroyed polyunsaturated fatty acid, in particular those associated with phospholipids (Weber et al., 2003). This affects the permeabilities of mitochondrial, endoplasmic reticulum, and plasma membranes, resulting in leakage of liver enzymes into the blood. Therefore, serum aminotransferase (ALT and AST) of CCl₄-intoxicated mice increased significantly. At the same time, the product of lipid peroxidation (MDA) was accumulated in CCl₄-damaged liver. An increase in MDA levels in the liver suggested enhanced peroxidation leading to tissue damage and failure of the antioxidant-defense mechanisms to prevent the formation of excessive free radicals (Naik, 2003). Administration with phenolic compound-enriched SCPE for 42 days could enhance the antioxidant capability, scavenge free radicals and prevent mitochondrial, endoplasmic reticulum, and plasma membranes from damage induced by free radicals. Therefore, it significantly reduced the activities of serum ALT and AST, and inhibited the formation of MDA in liver compared to the CCl₄-induced toxicity group.

We further studied the activities of two enzymes in the liver tissue in order to better understand mechanisms responsible for the hepatoprotective effects of SCPE in mice. SOD was an effective defense enzyme that catalyzes the dismutation of superoxide anions into hydrogen peroxide (H₂O₂) (Reiter et al., 2000). GSH-Px was an important enzyme catalyzed the reduction of H₂O₂ and hydroperoxides to non-toxic products and terminated the chain reaction of lipid peroxidation by removing lipid hydroperoxides from the cell membrane (Naik and Panda, 2007; Jung and Henke, 1996). Lipid peroxides or reactive oxygen species could easily inactivate these antioxidant enzymes in toxicity (Yang et al., 2008; Polavarapu et al., 1998). The results of the present study showed that SOD and GSH-Px activities were significantly decreased in the liver in response to CCl₄ treatment compared with control group mice, indicating increased oxidative damage to liver. However, SOD and GSH-Px activities were significantly elevated by administration of SCPE to CCl₄-intoxicated mice (Fig. 3B and C), suggesting that it has the ability to restore and maintain the activities of SOD and GSH-Px in CCl₄-damaged liver. Thus, administration of SCPE could effectively protect against the hepatic lipid peroxidation induced by CCl₄. The hepatoprotective effect of SCPE was also confirmed by the histopathological observations with shrinking sinusoidal, reducing inflammatory cell and ballooning degeneration.

Silymarin, an antioxidant flavonoid, had been used to treat hepatotoxicity diseases in clinical practice for at least two decades (Kren and Walterova, 2005; Shaker et al., 2010). Other phenolic compound, such as hesperetin (Tirkey et al., 2005), quercetin (Gilani et al., 1997; Lee et al., 2010) and caffeic acid phenethyl ester (Albukhari et al., 2009) also had been proved to possess hepatoprotective effect in mice against liver damage induced by lipid peroxidation. Moreover, a considerable amount of polyphenol extracts from natural products, such as propolis extract (Bhadauria et al., 2008), *Clerodendron glandulosum*. Coleb leaf extract (Jadeja et al., 2011), extract of *Murraya koenigii* L. (Desai et al., 2012), *Coriandrum sativum* extracts (Sreelatha et al., 2008) and apple polyphenols (Yang et al., 2010), had been investigated and confirmed the hepatoprotective effects. In fact, almost all of those studies demonstrated the mechanism by which phenolic compounds prevent against CCl₄-induced lipid peroxidation and hepatotoxicity was due to antioxidant activities. Thus, it was not surprising that the

same conclusion was deduced in this study. Higher total phenolic content and total flavonoid content (especially abundant Hesperetin and Quercetin) were observed in SCPE, and therefore remarkable high antioxidant activity, superior free radical scavenging ability and inhibiting lipid peroxidation contributed to hepatoprotective effect in mice against liver damage induced by CCl₄.

4. Conclusion

In conclusion, the results of this study demonstrate that SCPE is effective for the prevention of CCl₄-induced hepatic damage in mice and therefore it could be used as a hepatoprotective agent. The protective effects against acute liver damage may be, at least in part, due to the free radical scavenging effect, inhibition of lipid peroxidation, and increased antioxidant activity. This is the first report of the hepatoprotective effects of phenolic compound in pollen of *S. chinensis*. According to the results of this study, abundant flavonoid compounds (quercetin, galangin, hesperetin, resveratrol and kaempferol) are considered as the main hepatoprotective factor in SCPE. Further studies with individual active compounds existed in SCPE are underway which will enable us to understand the exact mechanism of hepatoprotective action by SCPE.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work is financially supported by The National Natural Science Foundation of China (No. 31272510), The Agricultural Science and Technology Project of Shaanxi Province (No. 2011K01-29) and The Science foundation of Xi'an Technology (NC10018 (18)).

References

- Afanas'ev, I.B., Dcrozshko, A.I., Brodskii, A.V., Kostyuk, V.A., Potapovitch, A.I., 1989. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem. Pharmacol.* 38, 1763–1769.
- Ai, J., Wang, Y.P., Li, C.Y., Yan, T.L., Guo, X.W., 2007. Pollen shape and pollination character of *Schisandra chinensis*. *J. Jilin Agr. Univ.* 29, 293–297.
- Albukhari, A.A., Gashlan, H.M., El-Beshbishy, H.A., Nagy, A.A., Abdel-Naim, A.B., 2009. Caffeic acid phenethyl ester protects against tamoxifen-induced hepatotoxicity in rats. *Food Chem. Toxicol.* 47, 1689–1695.
- Almaraz-Abarca, N., Campos, M.G., Ávila-Reyes, J.A., Naranjo-Jiménez, N., Herrera-Corral, J., González-Valdez, L.S., 2004. Variability of antioxidant activity among honeybee-collected pollen of different botanical origin. *Interciencia* 29, 574–578.
- Bhadauria, M., Nirala, S.K., Shukla, S., 2008. Multiple treatment of propolis extract ameliorates carbon tetrachloride induced liver injury in rats. *Food Chem. Toxicol.* 46, 2703–2712.
- Bonvehí, J.S., Torrentó, M.S., Lorente, E.C., 2001. Evaluation of polyphenolic and flavonoid compounds in honeybee-collected pollen produced in Spain. *J. Agr. Food Chem.* 49, 1848–1853.
- Brattin, W.J., Glende Jr., E.A., Recknagel, R.O., 1985. Pathological mechanisms in carbon tetrachloride hepatotoxicity. *J. Free Radical Biol. Med.* 1, 27–38.
- Brautbar, N., Williams II, J., 2002. Industrial solvents and liver toxicity: risk assessment, risk factors and mechanisms. *Int. J. Hyg. Environ Health* 205, 479–491.
- Campos, M., Markham, K.R., Mitchell, K.A., Cunha, P., 1997. An approach to the characterization of bee pollens via their flavonoid/phenolic profiles. *Phytochem. Anal.* 8, 181–185.
- Campos, M.G., Webby, R.F., Markham, K.R., Mitchell, K.A., da Cunha, A.P., 2003. Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. *J. Agric. Food Chem.* 51, 742–745.
- Chen, D.F., Zhang, S.X., Chen, K., Zhou, B.N., Wang, P., Cosentino, L.M., Lee, K.H., 1996. Two new lignans, interiotherins A and B, as anti-HIV principles from *Kadsura interior*. *J. Nat. Prod.* 59, 1066–1068.
- Chen, D.F., Zhang, S.X., Kozuka, M., Sun, Q.Z., Feng, J., Wang, Q., Mukainaka, T., Nobukuni, Y., Tokuda, H., Nishino, H., Wang, H.K., Morris-Natschke, S.L., Lee, K.H., 2002. Interiotherins C and D, two new lignans from *Kadsura interior* and antitumor-promoting effects of related neolignans on Epstein–Barr virus activation. *J. Nat. Prod.* 65, 1242–1245.

- Cheng, N., Wang, B.N., Deng, J.J., Gao, H., Cao, W., 2011. Antioxidant activities of ethanol extracts of rape pollen collected from different regions. *Food Sci.* 32, 34–36.
- Chu, Q.C., Tian, X.H., Jiang, L.M., Ye, J.N., 2007. Application of capillary electrophoresis to study phenolic profiles of honeybee-collected pollen. *J. Agric. Food Chem.* 55, 8864–8869.
- Desai, S.N., Patel, D.K., Devkar, R.V., Patel, P.V., Ramachandran, A.V., 2012. Hepatoprotective potential of polyphenol rich extract of *Murraya koenigii* L.: an in vivo study. *Food Chem. Toxicol.* 50, 310–314.
- Dudov, I.A., Starodub, N.F., 1994. Antioxidant system of rat erythrocytes under conditions of prolonged intake of honeybee flower pollen load. *Ukr. Biokhim. Zh.* 66, 94–96.
- Estevinho, L., Pereira, A.P., Moreira, L., Dias, L.G., Pereira, E., 2008. Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. *Food Chem. Toxicol.* 46, 3774–3779.
- Gilani, A.H., Janbaz, K.H., Shan, B.H., 1997. Quercetin exhibits hepatoprotective activities in rats. *Biochem. Soc. T.* 25, 29–31.
- Jadeja, R., Thounaojam, M., Ansarullah, Ramachandran, A.V., Devkar, R., 2009. Phytochemical constituents and free radical scavenging activity of *Clerodendron glandulosum* coleb methanolic extract. *J. Comp. Integr. Med.* 6, 19.
- Jadeja, R.N., Thounaojam, M.C., Ansarullah, Jadav, S.V., Patel, M.D., Patel, D.K., Salunke, S.P., Padate, G.S., Devkar, R.V., Ramachandran, A.V., 2011. Toxicological evaluation and hepatoprotective potential of *Clerodendron glandulosum* coleb leaf extract. *Hum. Exp. Toxicol.* 30, 63–70.
- Jia, Z., Tang, M., Wu, J., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radical. *Food Chem.* 64, 555–559.
- Jung, K., Henke, W., 1996. Developmental changes of antioxidant enzymes activity in kidney and liver from rats. *Free Radical Biol. Med.* 20, 613–617.
- Kren, V., Walterova, D., 2005. Silybin and silymarin – New effects and applications. *Biomed. Pap.* 149, 29–41.
- Kuo, Y.H., Li, S.Y., Huang, R.L., Wu, M.D., Huang, H.C., Lee, K.H., 2001. Schizarin B, C, D, and E, four new lignans from *Kadsura matsudai* and their antihepatitis activities. *J. Nat. Prod.* 64, 487–490.
- Lee, E.S., Lee, H.E., Shin, J.Y., Yoon, S., Moon, J.O., 2010. The flavonoid quercetin inhibits dimethylnitrosamine-induced liver damage in rats. *J. Pharm. Pharmacol.* 55, 1169–1174.
- Liang, Y., Cao, W., Chen, W.J., Xiao, X.H., Zheng, J.B., 2009. Simultaneous determination of four phenolic components in citrus honey by high performance liquid chromatography using electrochemical detection. *Food Chem.* 114, 1537–1543.
- Linskens, H.F., Jorde, W., 1997. Pollen as food and medicines – A review. *Econ. Bot.* 51, 77–78.
- Mărghitaș, L.A., Stanciu, O.G., Dezmirean, D.S., Bobiș, O., Popescu, O., Bogdanov, S., Campos, M.G., 2009. In vitro antioxidant capacity of honeybee-collected pollen of selected floral origin harvested from Romania. *Food Chem.* 115, 878–883.
- Naik, S.R., 2003. Antioxidants and their role in biological functions: an overview. *Indian Drugs* 40, 501–516.
- Naik, S.R., Panda, V.S., 2007. Antioxidant and hepatoprotective effects of *Ginkgo biloba* phytosomes in carbon tetrachloride-induced liver injury in rodents. *Liver Int.* 27, 393–399.
- Nandita, S., Rajini, P.S., 2004. Free radical scavenging activity of an aqueous extract of potato peel. *Food Chem.* 85, 611–616.
- Negri, G., Teixeira, E.W., Alves, M.L.T.M.F., Moreti, A.C.C.C., Otsuk, I.P., Borguini, R.G., Salatino, A., 2011. Hydroxycinnamic acid amide derivatives, phenolic compounds and antioxidant activities of extracts of pollen samples from Southeast Brazil. *J. Agric. Food Chem.* 59, 5516–5522.
- Polavarapu, R., Spitz, D.R., Sim, J.E., Follansbee, M.H., Oberiley, L.W., Rahemtulla, A., Nanji, A.A., 1998. Increased lipid peroxidation and impaired antioxidant enzyme function is associated with pathological liver injury in experimental alcoholic liver disease in rats fed diets high in corn oil and fish oil. *Hepatology* 27, 1317–1323.
- Recknagel, R.O., Glende Jr., E.A., Dolak, J.A., Waller, R.L., 1989. Mechanisms of carbon tetrachloride toxicity. *Pharmacol. Ther.* 43, 139–154.
- Reiter, R.J., Tan, D., Osuna, C., Gitto, E., 2000. Actions of melatonin in the reduction of oxidative stress. *J. Biomed. Sci.* 7, 444–458.
- Šarić, A., Balog, T., Sobočanec, S., Kušić, B., Šverko, V., Rusak, G., Likić, S., Bubalo, D., Pinto, B., Reali, D., Marotti, T., 2009. Antioxidant effects of flavonoid from *Croatian cystus incanus* L. rich bee pollen. *Food Chem. Toxicol.* 47, 547–554.
- Shaker, E., Mahmoud, H., Mnaa, S., 2010. Silymarin, the antioxidant component and *Silybum marianum* extracts prevent liver damage. *Food Chem. Toxicol.* 46, 803–806.
- Soares, J.R., Dins, T.C.P., Cunha, A.P., Almeida, L.M., 1997. Antioxidant activity of some extracts of *Thymus zygis*. *Free Radical Res.* 26, 469–478.
- Sreelatha, S., Padma, P.R., Umadevi, M., 2008. Protective effects of *Coriandrum sativum* extracts on carbon tetrachloride-induced hepatotoxicity in rats. *Food Chem. Toxicol.* 47, 702–708.
- Sun, C.R., 2000. Pollen morphology of the Schisandraceae and its systematic significance. *Acta Phytotax. Sin.* 38, 437–445.
- Sun, S.F., Chen, W.J., Cao, W., Zhang, F.Y., Song, J.R., Tian, C.R., 2008. Research on the chelation between quercetin and Cr (III) ion by Density Functional Theory (DFT) method. *J. Mol. Struct.* 860, 40–44.
- Tirkey, N., Pilkhwal, S., Kuhad, A., Chopra, K., 2005. Hesperidin, a citrus bioflavonoid, decreases the oxidative stress produced by carbon tetrachloride in rat liver and kidney. *BMC Pharmacol.* 5, 2.
- Wang, B.S., Huang, G.J., Tai, H.M., Huang, M.H., 2012. Antioxidant and anti-inflammatory activities of aqueous extracts of *Schizonepeta tenuifolia* Briq. *Food Chem. Toxicol.* 50, 526–531.
- Weber, L.W., Boll, M., Stampfl, A., 2003. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit. Rev. Toxicol.* 33, 105–136.
- Yang, X.W., Miyashiro, H., Hattori, M., Namba, T., Tezuka, Y., Kikuchi, T., Chen, D.F., Xu, G.J., Hori, T., Extine, M., 1992. Isolation of novel lignans, heteroclittins F and G, from the stems of *Kadsura heteroclita*, and anti-lipid peroxidative actions of heteroclittins A-G and related compounds in the in vitro rat liver homogenate system. *Chem. Pharm. Bull.* 40, 1510–1516.
- Yang, Y.S., Ahn, T.H., Lee, J.C., Moon, C.J., Kim, S.H., Jun, W., Park, S.C., Kim, H.C., Kim, J.C., 2008. Protective effects of Pycnogenol on carbon tetrachloride-induced hepatotoxicity in Sprague-Dawley rats. *Food Chem. Toxicol.* 46, 380–387.
- Yang, J.Y., Li, Y., Wang, F., Wu, C., 2010. Hepatoprotective effects of apple polyphenols on CCl₄-induced acute liver damage in mice. *J. Agric. Food Chem.* 58, 6525–6531.
- Yeh, Y.H., Hsieh, Y.L., Lee, Y.T., Hu, C.C., 2012. Protective effects of *Geloina eros* extract against carbon tetrachloride-induced hepatotoxicity in rats. *Food Res. Int.* 48, 551–558.
- Yıldırım, A., Mavi, A., Kara, A.A., 2001. Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. *J. Agric. Food Chem.* 49, 4083–4089.
- Zhang, H., Zhang, G.Q., Zhu, Z.Y., Zhao, L., Fei, Y., Jing, J., Chai, Y.F., 2009. Determination of six lignans in *Schisandra chinensis* (Turcz.) Baill. Fruits and related Chinese multiherb remedies by HPLC. *Food Chem.* 115, 735–739.
- Zhou, J., Li, P., Cheng, N., Gao, H., Wang, B.N., Wei, Y.H., Cao, W., 2012. Protective effects of buckwheat honey on DNA damage induced by hydroxyl radicals. *Food Chem. Toxicol.* 50, 2766–2773.