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## Black Soybean Shows Protective Function against Carbon Tetrachloride-induced Liver Damage in Sprague-dawely Rats

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### Research Article

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### ABSTRACT

Anthocyanins are abounding in the seed coat of black soybean (BS) (*Glycine max* (L.) Merrill), which contribute to anti-oxidative and anti-inflammatory activities. It led us to investigate the protective function of BS against a strong oxidant carbon tetrachloride (CCl<sub>4</sub>) induced liver damage in rats. BS was baked at 130 °C for 5 min and then was submerged in 100 °C hot water for 20 min to produce an anthocyanin enriched tea/decoction (BST). Protective function of BST against CCl<sub>4</sub>-induced liver damage in Sprague-Dawely rats was investigated with six experimental groups: control, high BST (1 g BS/kg bw), CCl<sub>4</sub> (0.5 ml 20% CCl<sub>4</sub>), CCl<sub>4</sub> + silymarin (0.2 g/kg bw), CCl<sub>4</sub> + low BST (0.1 g BS/kg bw) and CCl<sub>4</sub> + high BST (1 g BS/kg bw). BST enhanced GSH and GSSH contents, as well as antioxidant enzymes activities (superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities) in the liver tissue of normal rats. BST also attenuated the elevation of serum glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) levels in CCl<sub>4</sub> treated rats. Liver histopathology revealed that BST reduced fatty liver and liver fibrosis caused by CCl<sub>4</sub>. It was suggested that anthocyanins in the seed coat of black soybean contributed to such hepatoprotective effect.

### INTRODUCTION

Black soybean (*Glycine max* (L.) Merrill) is commonly used as medicinal food and herb in oriental countries. It has anti-inflammatory activity, antiproliferative effect, and antioxidant capacity, as well as the ability to reduce the risk of cancer [1-4]. The biological functions of black soybean are mainly associated with its anthocyanin compounds, which are abundant in the seed coat of black soybean [5-8]. It has been reported that anthocyanin extract from black rice bran has hepatoprotective effect against carbon tetrachloride-induced liver injury in mice and the extract from black rice can inhibit alcohol-induced chronically liver damage in rats [9,10]. Moreover, it is found that cyanidin-3-O-glucoside (C3G), an anthocyanin commonly found in many natural plants, showed hepatoprotective function in carbon tetrachloride treated mice [11]. Since the seed coat of black soybean contains high amount of anthocyanins and the major anthocyanin in the seed coat is C3G, it leads us to consider that black soybean may also exhibit such hepatoprotective functions. Thus, in this study, we evaluate the protective effect of black soybean on carbon tetrachloride-induced liver damage using Sprague-Dawely (SD) rats as the animal model [5].

Carbon tetrachloride (CCl<sub>4</sub>) is a strong oxidant for the production of chemical hepatic injury [12,13]. CCl<sub>4</sub> is transformed to trichloromethyl free radicals and these active free radicals are capable of attacking cellular macromolecules such as lipids, proteins, and DNA [14,15]. It has been reported that CCl<sub>4</sub> can decrease the antioxidant enzymes activities in the liver of rats, including superoxide dismutase, glutathione peroxidase, and glutathione reductase activities [16,17]. Intoxication with CCl<sub>4</sub> causes cellular necrosis, oxidative stress and inflammation, which leads to hepatic damages, such as fibrosis, cirrhosis, and atrophy [18].

Preheating treatments have been found to enhance the antioxidant activity in the extracts from black soybean. The water extract from black soybean baked at 130 °C for 5 min showed higher total anthocyanin content and higher antioxidant activity than the water extract from un-preheated black soybean [19]. Such preheating processes are considered to increase the extractability of anthocyanin from black soybean. In this study, pre-baked (at 130 °C for 5 min) whole black soybean was submerged in 100 °C hot water for 20 min to produce a decoction. The liver protective function of the black soybean decoction was evaluated using carbon tetrachloride treated SD rats.

## MATERIALS AND METHODS

### Chemicals

Carbon tetrachloride (CCl<sub>4</sub>) and silymarin were purchased from Sigma Co. (St. Louis, MO). Glutathione disulfide (GSSG), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione (GSH) and N-ethylmaleimide were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hydrogen peroxide and cumene hydroperoxide were purchased from Alfa Aesar (Heysham, UK). Total protein biuret reagent was purchased from Thermo (Rockford, IL, USA).

### Preparation of Black Soybean Tea (BST)

Black soybean (*Glycine max* (L.) Merrill, Tainan No. 3) was purchased from a local farmer in Chiayi county, Taiwan, ROC. After washing, whole black soybean was put in an aluminum pan (20\*15\*1.5 cm) and preheated in an oven (model SO-1199, Sunpentown, Taipei, Taiwan) at 130 °C for 5 min. A digital pocket thermometer (model DGS-A9SA-ST9215C, Dogger Co., Taipei, Taiwan) was used to monitor the environmental temperature around the black soybean. Preheated black soybean was then submerged in 10 times (w/v) of 100 °C hot distilled water for 20 min. After removing the bean, the decoction was served as the sample for animal treatments.

### Experimental Animals

Six-month old male Sprague-Dawley (SD) rats were purchased from Biolasco Taiwan Co., Ltd. (I-Lan, Taiwan). The rats were housed in stainless wire-bottom cages in an environmentally controlled room (22 ± 2 °C, 65 ± 5% relative humidity) with a 12 hr night and dark cycle. During the experiment, the rats were fed with a regular diet (Fu-So pellet chow, Taichung, Taiwan) and water ad libitum. The body weights were recorded weekly. This study protocol was approved by the Animal Research Ethics Committee at Providence University, Taichung, and Taiwan ROC.

Forty-eight rats were randomly divided into six groups, eight rats each. They were: control (the regular diet), high BST (1 g BS/kg bw), CCl<sub>4</sub> (0.5 ml 20% CCl<sub>4</sub>/olive oil), CCl<sub>4</sub> + silymarin (0.5 ml 20% CCl<sub>4</sub>/olive oil + 0.2 g silymarin/kg bw), CCl<sub>4</sub> + low BST (0.5 ml 20% CCl<sub>4</sub>/olive oil + BST at 0.1 g BS/kg bw) and CCl<sub>4</sub> + high BST (0.5 ml 20% CCl<sub>4</sub>/olive oil + BST at 1 g BS/kg bw). For the groups that CCl<sub>4</sub> was required (CCl<sub>4</sub> + silymarin, CCl<sub>4</sub> + low BST and CCl<sub>4</sub> + high BST), the rats received an intraperitoneal injection of 0.5 mL CCl<sub>4</sub> (20% CCl<sub>4</sub>/olive oil) twice a week for 9 weeks. The control and CCl<sub>4</sub> group received distilled water every day for 9 weeks; while high BST, CCl<sub>4</sub> + silymarin, CCl<sub>4</sub> + low BST and CCl<sub>4</sub> + high BST groups received BST (1 g BS/kg bw), silymarin (0.2 g/kg bw), BST (0.1 g BS/kg bw) and BST (1 g BS/kg bw) everyday, respectively.

At week 1, 3, 6 and 9, blood samples were collected in blood tube and then were centrifuged at 4 °C for 10 min. After centrifugation, the supernatant were collected for blood biochemical analyses. After 9 weeks, the rats were fasted for 24 h and sacrificed via cervical dislocation. The livers were immediately collected, washed and weighted. For antioxidant enzyme activity measurements, 1 g of liver tissue was homogenized with 1 ml 0.05 M phosphate buffer (pH 7.4) at 4 °C, 13000 rpm for 10 min. The dissected livers were immediately fixed in 6% formaldehyde for specimen preparation and histochemical staining analysis.

### Measurement of Superoxide Dismutase (SOD) Activity

The superoxide dismutase (SOD) activity was measured using the method reported by Marklund and Marklund with a slight modification [20]. One hundred microliters of the liver supernatant was mixed with 1 mL of a 0.05 M Tris-HCl and 7 µl of 150 mM pyrogallol, and then incubated at 25 °C for 10 min, then the activity was measured at 325 nm with a spectrophotometer (model U-2800, Hitachi). One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%.

### Measurement of Catalase (CAT) Activity

The CAT assay was carried out by the method reported by Aebi [21]. Liver homogenate from all groups were taken with 1.9 ml of phosphate buffer (pH 7.4), and the reaction was initiated by adding of 1 ml of H<sub>2</sub>O<sub>2</sub> (30 mM). Blank without liver homogenate was prepared with 2.9 ml of phosphate buffer and 1 ml of H<sub>2</sub>O<sub>2</sub>. The decrease in optical density due to the decomposition of H<sub>2</sub>O<sub>2</sub> was measured at the end of 1 min against the blank at 240 nm.

### Measurement of Glutathione Peroxidase (GPx) Activity

The activity of glutathione peroxidase was measured by a modification of the method of Paglia and Valentine [22]. The oxidation of NADPH was recorded as the decrease in absorbance at 340 nm with a spectrophotometer (model U-2800, Hitachi). The assay mixture contained 620 µl 0.25 M phosphate buffer (pH 7.4), 200 µl 5 unit/ml GR, 50 µl 40 mM GSH, 100 µl liver supernatant, 10 µl 20 mM NADPH and 20 µl 15 mM cumene hydroperoxide.

### Measurement of Glutathione Reductase (GR) Activity

Determination of GR activity was performed by a modification of the method of Paglia and Valentine [22]. The assay mixture contained 30  $\mu$ l liver homogenate, 750  $\mu$ l GSSG and 150  $\mu$ l NADPH, then the activity was measured at 340 nm with a spectrophotometer (model U-2800, Hitachi).

### Determination of GSH and GSSG Contents

GSH content was determined by a modification of the method of Hissin and Hilf [23]. To 0.5 ml of the 10,000 g supernatant, 4.5 ml of the phosphate-EDTA buffer, pH 8.0, was added. The final assay mixture (2.0 ml) contained 100  $\mu$ l of the diluted tissue supernatant, 1.8 ml of phosphate-EDTA buffer, and 100  $\mu$ l of the OPT solution, containing 100  $\mu$ g of OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette. Fluorescence at 420 nm was determined with the activation at 350 nm.

GSSH content was also determined using the method reported by Hissin and Hilf [23]. A 0.5 ml portion of the original 10,000 g supernatant was incubated at room temperature with 200  $\mu$ l of 0.04 M NEM for 30 min to interact with GSH present in the tissue. To this mixture, 4.3 ml of 0.1 N NaOH was added. A portion of this mixture (100  $\mu$ l) was taken for measurement of GSSG, using the procedure outlined above for GSH assay, except that 0.1 N NaOH was employed as diluent rather than phosphate-EDTA buffer.

### Determination of Malondialdehyde (MDA) Content

MDA determination was performed according to the method of Tatum et al. [24]. To liver homogenate, 0.4% TBA and 75  $\mu$ l 0.2% BHT was added, and then the mixture was vigorously agitated in a vortex and placed in a boiling water bath for 45 min. After cooling, to 500  $\mu$ l of the 10,000 g supernatant, 1000  $\mu$ l n-butanol was added. After thorough mixing and incubation at room temperature for 10 min, the mixture was centrifuged at 10,000 g for 10 min. The supernatant was transferred to a quartz cuvette. Fluorescence at 550 nm was determined with the activation at 515 nm.

### Determination of tissue protein concentration

The tissue protein concentration was estimated according to the method of Lowry et al using bovine serum albumin as the standard [25].

### Histopathology Analysis

The liver samples were paraffin-embedded for histological analysis. Hematoxylin-eosin (HE) stain was performed.

### Statistical Analysis

One-way analysis of variance (one-way ANOVA) was conducted using a package (SAS Institute Inc., Cary, NC). Duncan's multiple ranges test was used to determine the significant difference between different treatments.

## RESULTS AND DISCUSSION

### The Effects of CCl<sub>4</sub> and BST on the Body Weight and Liver Weight of SD Rats

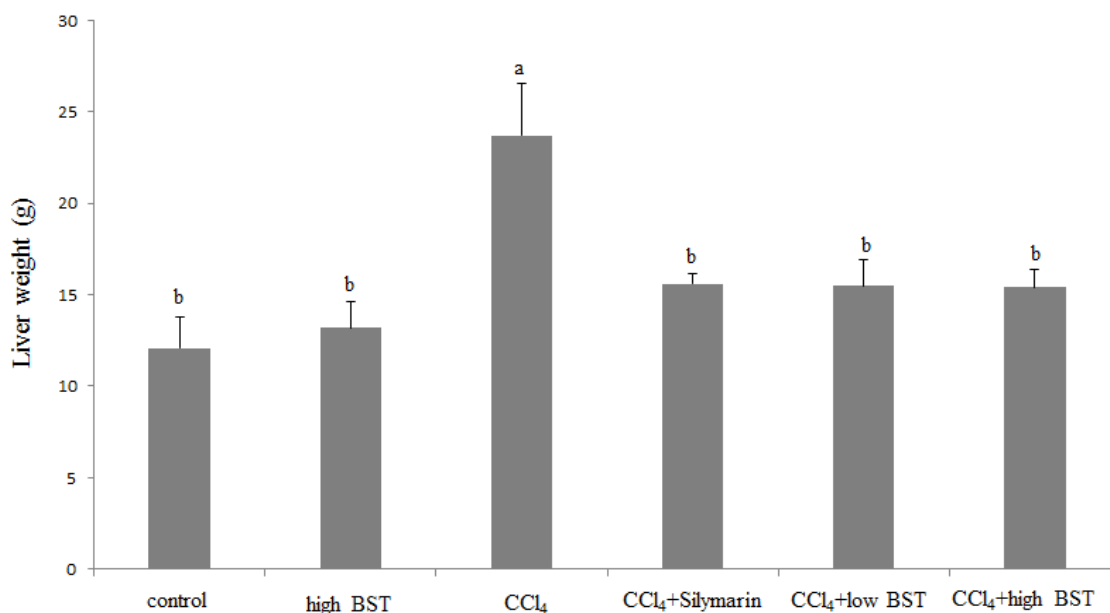
**Figure 1A** shows the change of body weight in SD rats during 9 weeks experimental period. It was found that CCl<sub>4</sub> group had significantly lower body weight than the control group at 3 week and after. In this study, CCl<sub>4</sub> was used as a strong oxidant to induce liver damage in SD rats. Therefore, the decline of the body weight was considered as the damage induced by CCl<sub>4</sub>. Hsu et al. used 8% CCl<sub>4</sub> (1 ml CCl<sub>4</sub>/kg bw) to induce liver damage in male Wistar rats and they found no significant difference in the body weight between CCl<sub>4</sub> treated and normal rats [18]. However, Li et al. and Lin et al. stated that CCl<sub>4</sub> significantly decreased the body weight of male Wistar rats [26,27]. In our case, we found that CCl<sub>4</sub> treated SD rats had diarrhea problem which might result in the decline of body weight. The administration of BST at 1 g BS/kg bw alone or co-treatment of BST at 0.1 g BS/kg bw or 1 g BS/kg bw with CCl<sub>4</sub> resulted in similar body weight profile with the control group, indicating that co-treatment of BST could reduce the damage caused by CCl<sub>4</sub>.

At the end of 9 weeks experimental period, SD rats were sacrificed and the liver tissues were collected for testing. The liver weights of SD rats increased significantly by CCl<sub>4</sub> treatment. It was found that the liver weight in CCl<sub>4</sub> group was about twice of the control group (**Figure 1B**). Our data indicated that co-treatment of BST at 0.1 g BS/kg bw or 1 g BS/kg bw with CCl<sub>4</sub> decreased the liver weight, and showed no significant difference with the control group.

### The Effects of CCl<sub>4</sub> and BST on GOT and GPT Levels of SD Rats

Direct evidences of liver damage were associated with the increases of serum GOT and GPT levels in CCl<sub>4</sub> treated SD rats. Compare to the control group, GOT level in CCl<sub>4</sub> group increased 26%, 66%, 223% and 402% at week 1, 3, 6 and 9, respectively (**Table 1**). GPT level in CCl<sub>4</sub> group also increased 26%, 74%, 275% and 805% at week 1, 3, 6 and 9, respectively. The data indicated that CCl<sub>4</sub> induced severe liver damage in SD rats at week 6 and 9. However, co-treatment of BST at 0.1 g BS/kg bw or 1 g BS/kg

bw completely recovered both the GOT and GPT levels; there were no significant differences ( $p > 0.05$ ) in those levels between the co-treatment group and the control group. The recovery effect of BST was similar to a well known liver protective agent, silymarin, at 0.2 g/kg bw in  $CCl_4$  treated SD rats.



**Figure 1.** Changes of body weight (A) in SD rats fed with different experimental diets for 9 weeks (◆ control, ■ high BST (1 g BS/kg bw), ▲  $CCl_4$ , ✕  $CCl_4$ +Silymarin (0.2 g/kg bw), ✱  $CCl_4$ + low BST (0.1 g BS/kg bw), ●  $CCl_4$ + high BST (1 g BS/kg bw) and the liver weight (B) of SD rats in week 9.

**Table 1.** Effect of BST on the activities of serum GOT and GPT in  $CCl_4$ -treated rats at 1, 3, 6 and 9 weeks.

Treatments	GOT (U/L)			
	Week 1	Week 3	Week 6	Week 9
control	141.4 ± 10.05 <sup>c</sup>	134.0 ± 5.03 <sup>b</sup>	130.6 ± 13.70 <sup>b</sup>	97.1 ± 9.75 <sup>b</sup>
high BST	133.9 ± 9.34 <sup>c</sup>	121.0 ± 11.25 <sup>b</sup>	126.1 ± 10.02 <sup>b</sup>	76.6 ± 6.53 <sup>b</sup>
$CCl_4$	178.6 ± 12.62 <sup>a</sup>	222.3 ± 40.79 <sup>a</sup>	420.6 ± 107.15 <sup>a</sup>	487.7 ± 76.32 <sup>a</sup>
$CCl_4$ +Silymarin	148.3 ± 14.28 <sup>bc</sup>	138.4 ± 5.29 <sup>b</sup>	151.0 ± 5.16 <sup>b</sup>	103.3 ± 13.14 <sup>b</sup>
$CCl_4$ +low BST	159.9 ± 27.72 <sup>b</sup>	138.6 ± 7.09 <sup>b</sup>	147.9 ± 7.90 <sup>b</sup>	105.7 ± 11.21 <sup>b</sup>
$CCl_4$ +high BST	141.1 ± 7.99 <sup>c</sup>	139.7 ± 28.56 <sup>b</sup>	149.0 ± 7.05 <sup>b</sup>	104.0 ± 10.39 <sup>b</sup>

Treatments	GPT (U/L)			
	Week 1	Week 3	Week 6	Week 9
control	49.9 ± 9.92 <sup>b</sup>	43.6 ± 3.69 <sup>c</sup>	45.9 ± 3.89 <sup>b</sup>	34.3 ± 2.14 <sup>b</sup>
high BST	47.1 ± 7.20 <sup>b</sup>	43.0 ± 3.61 <sup>c</sup>	41.9 ± 4.45 <sup>b</sup>	32.1 ± 2.41 <sup>b</sup>
$CCl_4$	62.6 ± 8.79 <sup>a</sup>	75.1 ± 19.66 <sup>a</sup>	169.6 ± 57.87 <sup>a</sup>	308.6 ± 47.82 <sup>a</sup>
$CCl_4$ +Silymarin	52.0 ± 12.69 <sup>ab</sup>	61.0 ± 10.58 <sup>b</sup>	58.1 ± 6.20 <sup>b</sup>	43.3 ± 5.71 <sup>b</sup>
$CCl_4$ +low BST	53.6 ± 9.16 <sup>ab</sup>	61.0 ± 10.39 <sup>b</sup>	54.4 ± 8.50 <sup>b</sup>	44.9 ± 7.13 <sup>b</sup>
$CCl_4$ +high BST	53.3 ± 8.79 <sup>ab</sup>	63.3 ± 11.87 <sup>ab</sup>	52.3 ± 4.11 <sup>b</sup>	45.6 ± 7.89 <sup>b</sup>

All values are expressed as mean ± SD. n=8

<sup>a-c</sup>Means in the same column followed by different letters are significantly different ( $p < 0.05$ )

### The Effects of $CCl_4$ and BST on Blood Lipids of SD Rats

The effects of  $CCl_4$  and BST on serum triglyceride and cholesterol levels in SD rats are shown in **Table 2**. Compared to the control group,  $CCl_4$  caused 32% decrease in the triglyceride level at week 9. It was possible that  $CCl_4$  reduced the formation of very low-density lipoprotein (VLDL) in endoplasmic reticulum of the liver, thus it prevented the transfer of triglyceride to blood and resulted low serum triglyceride level in the blood system [28]. The co-treatment of BST at 0.1 g BS/kg bw or 1 g BS/kg bw recovered the triglyceride level in  $CCl_4$  treated SD rats. Hsu et al. also found that  $CCl_4$  reduced 28% triglyceride level in male Wistar rats [16]. However, our data showed that  $CCl_4$  did not differ cholesterol level in SD rats. BST also showed no significant effect on the cholesterol level in normal or  $CCl_4$  treated SD rats.

**Table 2.** Effect of BST on the activities of serum cholesterol and triglyceride in  $CCl_4$ -treated rats at 1, 3, 6 and 9 weeks.

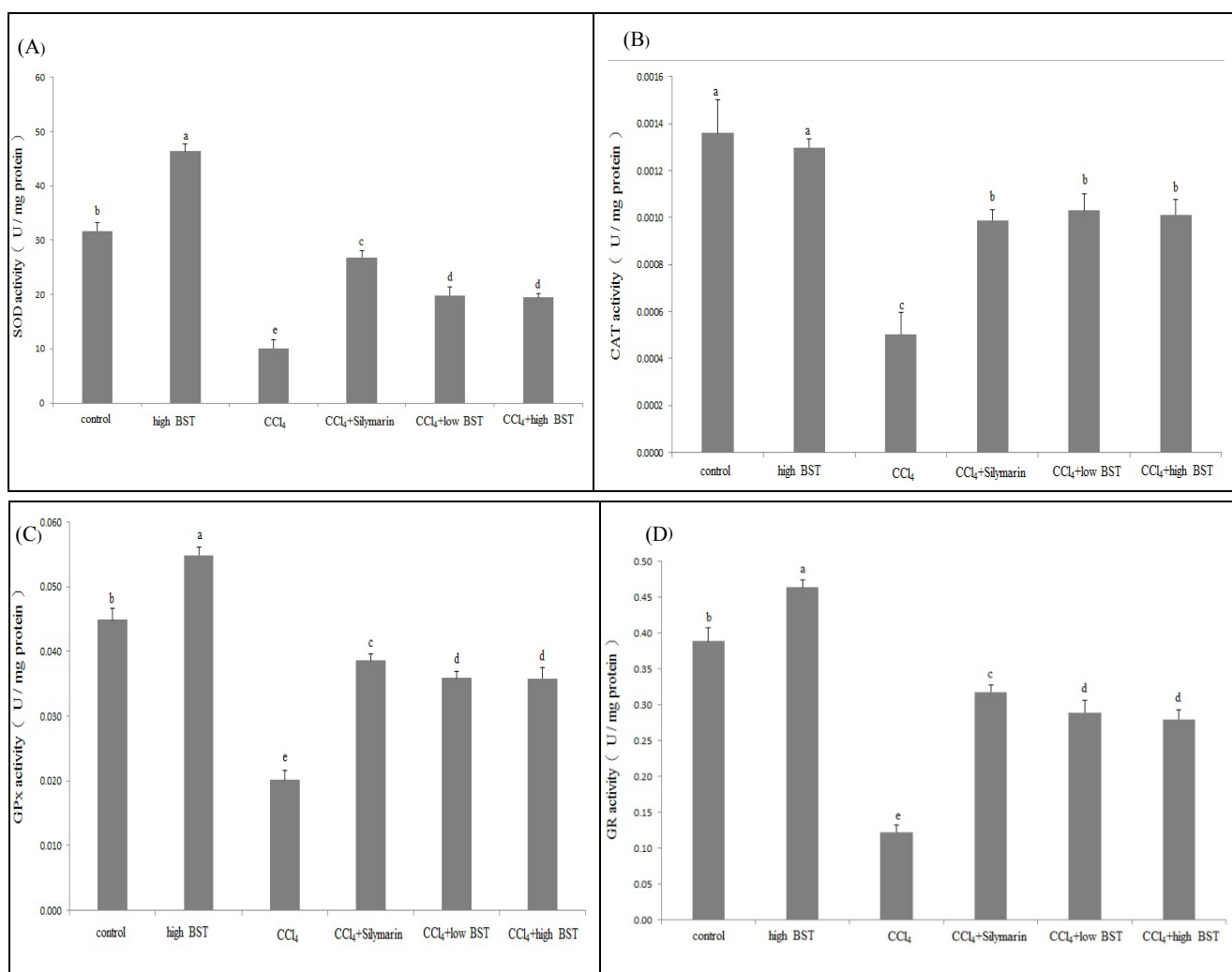
Treatments	Cholesterol (mg/dL) <sup>1</sup>			
	Week 1	Week 3	Week 6	Week 9
control	85.5 ± 11.05	75.4 ± 8.19	68.5 ± 5.98	61.0 ± 11.34
high BST	94.4 ± 13.65	81.8 ± 11.07	73.8 ± 14.85	59.9 ± 5.17

CCl <sub>4</sub>	85.3 ± 17.77	75.5 ± 10.03	70.4 ± 23.26	73.9 ± 13.38
CCl <sub>4</sub> +Silymarin	91.1 ± 18.60	74.9 ± 6.10	61.3 ± 7.25	66.9 ± 6.77
CCl <sub>4</sub> +low BST	95.8 ± 13.13	76.3 ± 6.32	69.6 ± 10.93	70.3 ± 13.02
CCl <sub>4</sub> +high BST	84.1 ± 15.16	74.9 ± 11.95	67.6 ± 4.34	64.4 ± 12.15
<b>Treatments</b>	<b>Triglyceride (mg/dL)</b>			
	<b>Week 1</b>	<b>Week 3</b>	<b>Week 6</b>	<b>Week 9</b>
control	107.9 ± 54.46 <sup>a</sup>	135.6 ± 101.86 <sup>a</sup>	104.0 ± 17.55 <sup>a</sup>	76.8 ± 16.45 <sup>ab</sup>
high BST	101.8 ± 34.69 <sup>ab</sup>	141.6 ± 43.06 <sup>a</sup>	112.8 ± 10.81 <sup>a</sup>	86.8 ± 5.39 <sup>a</sup>
CCl <sub>4</sub>	72.3 ± 17.62 <sup>ab</sup>	95.3 ± 45.27 <sup>ab</sup>	102.9 ± 18.67 <sup>ab</sup>	52.4 ± 9.10 <sup>c</sup>
CCl <sub>4</sub> +Silymarin	101.4 ± 42.93 <sup>ab</sup>	87.3 ± 21.48 <sup>ab</sup>	90.4 ± 9.59 <sup>bc</sup>	68.9 ± 19.33 <sup>b</sup>
CCl <sub>4</sub> +low BST	67.8 ± 19.83 <sup>b</sup>	57.0 ± 16.52 <sup>b</sup>	80.3 ± 6.88 <sup>c</sup>	63.3 ± 15.40 <sup>bc</sup>
CCl <sub>4</sub> +high BST	75.6 ± 25.15 <sup>ab</sup>	64.0 ± 14.71 <sup>b</sup>	90.8 ± 2.43 <sup>bc</sup>	67.0 ± 13.16 <sup>bc</sup>

All values are expressed as mean ± SD. n=8  
<sup>‡</sup>All values in the same column are not significantly different (p>0.05)  
<sup>a-c</sup>Means in the same column followed by different letters are significantly different (p<0.05)

### The Effects of CCl<sub>4</sub> and BST on Antioxidant Status of SD Rats

SD rats with the administration of BST alone at 1 g BS/kg bw had higher SOD, GPx and GR activities in the liver tissue than the control group, but no significant difference was found in the catalase activity (**Figure 2A-2D**). However, CCl<sub>4</sub> treatment declined SOD, GPx, GR and catalase activities in the liver tissue. Co-treatment of BST at 0.1 g BS/kg bw or 1 g BS/kg bw increased all antioxidant enzymes activities in the liver tissue of CCl<sub>4</sub> treated SD rats. The data indicated that the administration of BST enhanced antioxidant enzymes activities in both normal and CCl<sub>4</sub> treated liver tissue of SD rats.

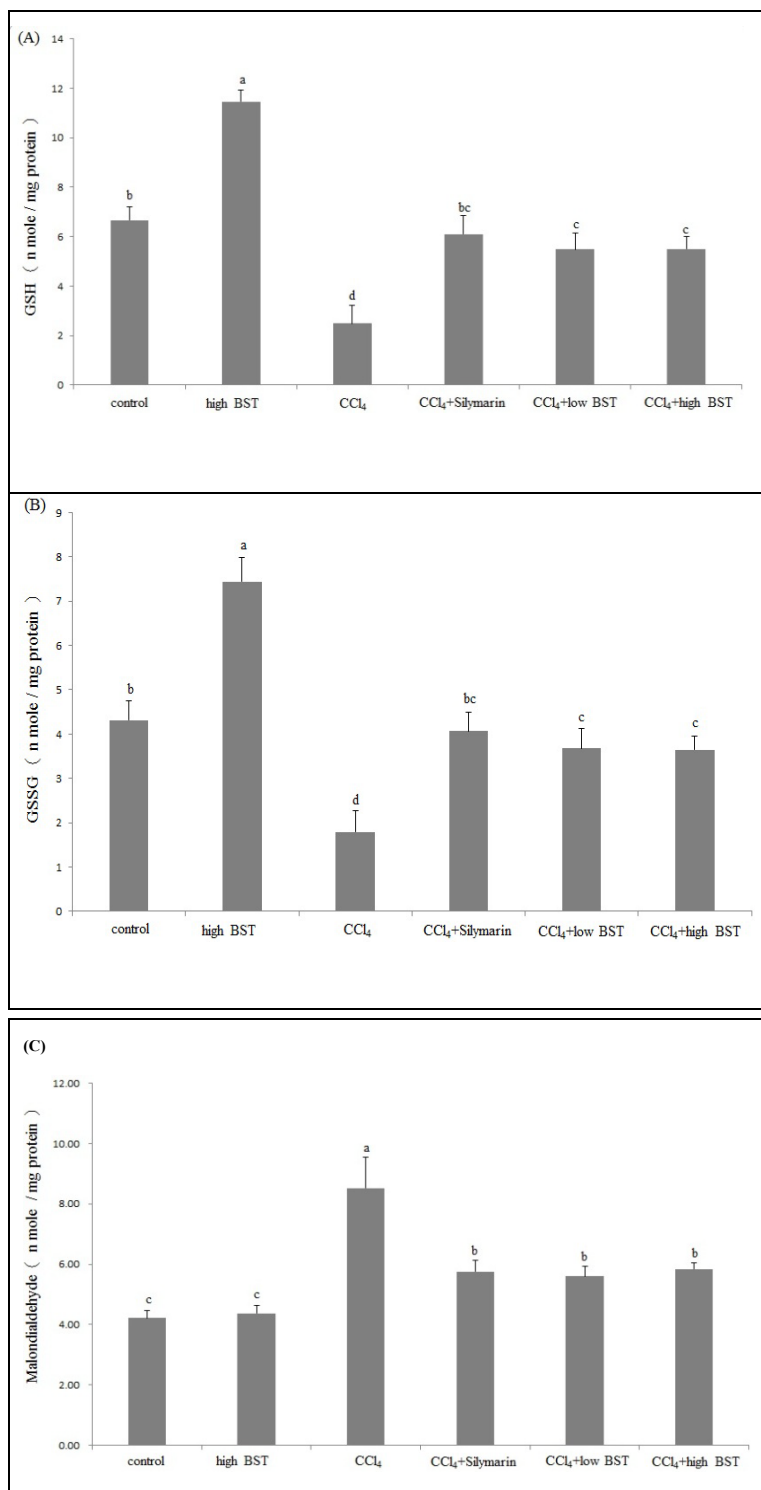


**Figure 2.** Superoxide dismutase (SOD) activity (A), catalase (CAT) activity (B), glutathione peroxidase (GPx) activity (C) and glutathione reductase (D) (GR) activity in the liver tissue of normal and liver-damaged SD rats fed with different experimental diets for 9 weeks.

GSH and GSSG contents in liver tissue of SD rats are shown in **Figure 3A and 3B** respectively. The administration of BST alone at 1 g BS/kg bw significantly increased both GSH and GSSG contents in the liver tissue than the control group. The rates of

increase were 77% and 72% for GSH and GSSG, respectively. Using  $\text{CCl}_4$  to induce liver damage, it was found that both GSH and GSSG contents in  $\text{CCl}_4$  group were only about 31% of the control group. Compare to  $\text{CCl}_4$  group, the co-treatment of BST showed significant higher GSH and GSSG levels in the liver, regardless the concentration of 0.1 g BS/kg bw or 1 g BS/kg bw. The data indicated that the administration of BST alone enhanced both GSH and GSSG contents in the liver, BST could also raise both GSH and GSSG contents in the liver tissue of  $\text{CCl}_4$  treated SD rats. It is well known that both GSH and GSSG played important roles in the defense system for oxidative stress; our results indicated that BST could enhance the antioxidant status in the liver tissue of SD rats.

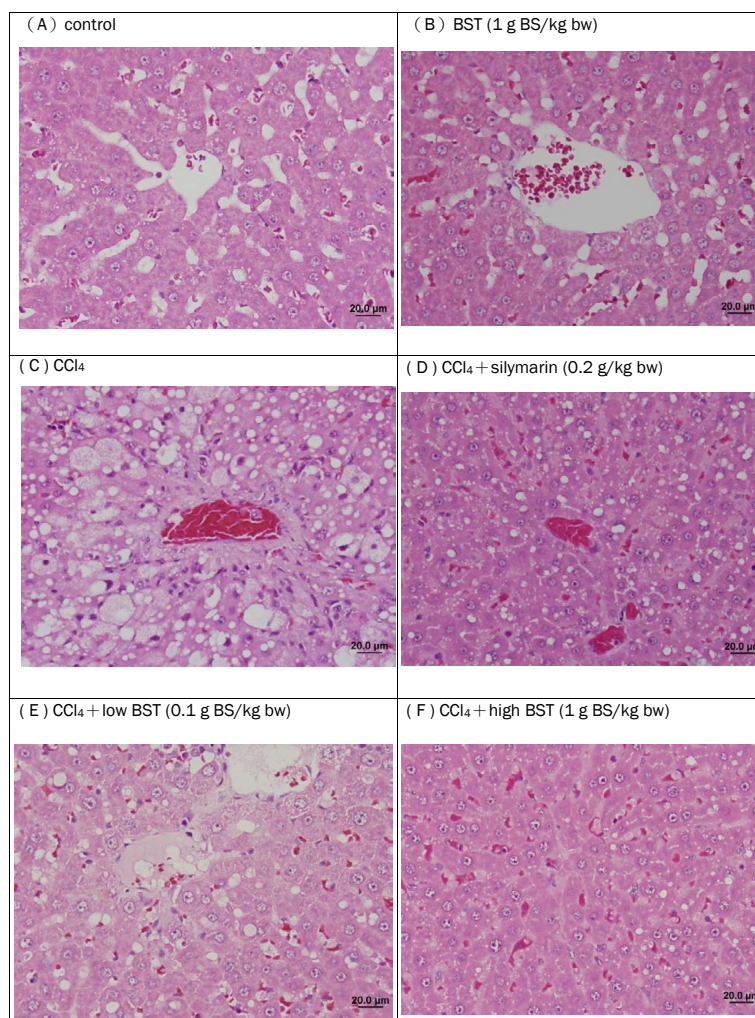
The free radicals derived from  $\text{CCl}_4$  might cause peroxidation of the membrane lipid and subsequently disrupt membrane integrity<sup>[19]</sup>. Measurement of malondialdehyde content is often used as an index for lipid peroxidation in cell or animal model. The malondialdehyde level in  $\text{CCl}_4$  group was twice of that in the control group (**Figure 3C**). While, co-treatment of BST at 0.1 g BS/kg bw or 1 g BS/kg bw significantly reduced the malondialdehyde level in the liver tissue of  $\text{CCl}_4$  treated SD rats.



**Figure 3.** GSH content (A), GSSG content (B) and malondialdehyde (MDA) content (C) in the liver tissue of normal and liver-damaged SD rats fed with different experimental diets for 9 weeks.

### The Effects of CCl<sub>4</sub> and BST on the Liver Histopathology of SD Rats

Liver histopathology revealed that liver sections from the control and BST treated rats showed normal liver architecture, but CCl<sub>4</sub> induced serious fatty liver and liver fibrosis in SD rats (**Figure 4**). Co-treatment of BST at 0.1 g BS/kg bw or 1 g BS/kg bw prevented the development of histopathological changes induced by CCl<sub>4</sub>. The percentages of fat accumulation in the liver for the control group and BST alone groups were 0%, for CCl<sub>4</sub> group was 33~66%, and for co-treatment of silymarin or BST at 0.1 g BS/kg bw or 1 g BS/kg bw was less than 10%. The data indicated that CCl<sub>4</sub> group showed serious fatty liver, while co-treatment of silymarin or BST group had slight fatty liver. Metavir score revealed that the level of liver fibrosis in CCl<sub>4</sub> treated SD rats was rigid, while the other groups did not show any sign of fibrosis.



**Figure 4.** Sections of the liver of SD rats staining with Hematoxylin-eosin (HE) (×600).

Our results showed that co-treatment of BST could reduce the lipid peroxidation, enhance the antioxidant enzyme activities, recover serum GOT and GPT levels, decrease the liver weight and increase the body weight in CCl<sub>4</sub> treated SD rats. Histopathology analysis also showed that co-treatment of BST could significantly recover the fatty liver and liver fibrosis in CCl<sub>4</sub> treated SD rats. It demonstrated, for the first time, that BST could reduce the liver damage caused by CCl<sub>4</sub>. Treatment of BST alone also enhanced GSH and GSSH contents, as well as antioxidant enzymes activities (SOD, GPx and GR activities) in the liver tissue, while maintained normal liver weight and body weight, normal GOT and GPT levels and normal liver architecture. In general, the protective effect of BST was similar to that of silymarin at the level of 0.2 g/kg bw. Therefore, BST could be considered as a potential liver protective agent.

It is worth to mention that BST was prepared simply by immersing preheated black soybean in 100°C hot water for 20 min. The concentrations of BST used in this study were 0.1 g BS/kg bw and 1 g BS/kg bw, and the amount of BST was based on the weight of whole black soybean, not the weight of the extract from black soybean. It is possible that the extracts from black soybean may have better liver protective function than BST; in other words, lower concentrations may be enough for the extracts to achieve the same effect as BST. Therefore, the application of proper extraction techniques to obtain the active compounds in black soybean is important and requires further investigation.

Anthocyanins are the main biological compounds in the seed coat of black soybean currently receiving many attentions. And cyanidin-3-O-glucoside (C3G) is the main anthocyanin in the seed coat of black soybean [12]. It has been reported that administration of a pure C3G attenuates the levels of serum aspartate aminotransferase and alanine aminotransferase and

reduces the incidence of liver lesions in CCl<sub>4</sub>-treated mice. C3G apparently protects the liver from CCl<sub>4</sub>-induced hepatic damage through antioxidant and anti-inflammatory mechanisms [11]. In this study, BST shows significant protective effect against CCl<sub>4</sub> induced liver damage in rats. BST could release CCl<sub>4</sub> induced oxidative stress by enhancing antioxidant enzymes activities. And this may be due to the antioxidant activity of C3G.

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### REFERENCES

1. Kim JM, et al. Effects of black soybean [*Glycine max* (L.) Merr.] seed coats and its anthocyanidins on colonic inflammation and cell proliferation in vitro and in vivo. *J Agric Food Chem*. 2008;56:8427-8433.
2. Nizamutdinova IT, et al. Anthocyanins from black soybean seed coats preferentially inhibit TNF-alpha-mediated induction of VCAM-1 over ICAM-1 through the regulation of GATAs and IRF-1. *J Agric Food Chem*. 2009;57:7324-7330.
3. Kim JA, et al. A correlation between the level of phenolic compounds and the antioxidant capacity in cooked-with-rice and vegetable soybean (*Glycine max* L.) varieties. *Eur Food Res Technol*. 2006;224:259-270.
4. Do MH, et al. Intake of fruits, vegetables, and soy foods in relation to breast cancer risk in Korean women: a case-control study. *Nutr Cancer*. (2007);57:20-27.
5. Choung MG, et al. Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.). *J Agric Food Chem*. 2001;49:5848-5851.
6. Furuta S, et al. Radical-scavenging activities of soybean cultivars with black seed coats. *Food Sci Technol Res*. 2003; 9:73-75.
7. Astadi IR, et al. In vitro antioxidant activity of anthocyanins of black soybean seed coat in human low density lipoprotein (LDL). *Food Chem*. 2008;112:659-663.
8. Lee JH, et al. Characterisation of anthocyanins in the black soybean (*Glycine max* L.) by HPLC-DAD-ESI/MS analysis. *Food Chem*. 2009;12:226-231.
9. Hou F, et al. Hepatoprotective and antioxidant activity of anthocyanins in black rice bran on carbon tetrachloride-induced liver injury in mice. *J Funct Foods*. 2013;5:1705-1713.
10. Hou Z, et al. Effect of anthocyanin-rich extract from black rice (*Oryza sativa* L. Japonica) on chronically alcohol-induced liver damage in rats. *J Agric Food Chem*. 2010; 58: 3191-3196.
11. Cho BO, et al. Cyanidin-3-glucoside ameliorates CCl<sub>4</sub>-induced liver injury in mice. *Food Sci Biotechnol*. 2014;23:1313-1319.
12. Kujawska M, et al. Protective effect of red beetroot against carbon tetrachloride- and N-nitrosodiethylamine-induced oxidative stress in rats. *J Agric Food Chem*. 2009;57:2570-2575.
13. Hsu YW, et al. Protective effects of *Dunaliella salina*—a carotenoids-rich alga, against carbon tetrachloride-induced hepatotoxicity in mice. *Food Chem Toxicol*. 2008;46:3311-3317.
14. Campo GM, et al. Hyaluronic acid and chondroitin-4-sulphate treatment reduces damage in carbon tetrachloride-induced acute rat liver injury. *Life Sci*. 2004;74:1289-1305.
15. Hsu YW, et al. Protective effects of silica hydride against carbon tetrachloride-induced hepatotoxicity in mice. *Food Chem Toxicol*. 2010;48:1644-1653.
16. Hassan MH, et al. Antioxidant and antiapoptotic effects of capsaicin against carbon tetrachloride-induced hepatotoxicity in rats. *Toxicol Ind Health*. 2011;28:248-438.
17. Gomes A, et al. Hepatoprotective activity of the edible snail (*Bellamia bengalensis*) flesh extract in carbon tetrachloride induced hepatotoxicity in rats. *J Ethnopharmacol*. 2011;38:228-232.
18. Hsu LS, et al. Mulberry water extracts (MWEs) ameliorated carbon tetrachloride-induced liver damages in rat. *Food Chem Toxicol*. 2012;50:3086-3093.
19. Hsu CK, et al. Influence of preheating on antioxidant activity of the water extract from black soybean and color and sensory properties of black soybean decoction. *J Sci Food Agric*. 2013;93:3883-3890.
20. Marklund S and Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem*. 1974;47:469-474.
21. Aebi H. *Catalase: Methods of Enzymatic Analysis*. Weinheim Deerfield Beach, Florida (1983).
22. Paglia DE and Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med*. 1967;70:158-169.



23. Hissin PJ and Hilf R A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem.* 1976;74:214-226.
24. Tatum VL, et al. Measurement of malondialdehyde by high performance lipid chromatography with fluorescence detection. *Lipids.* 1990;25:226-229.
25. Lowry Oh, et al. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193:265-275.
26. Li R, et al. Puerarin mediates hepatoprotection against CCl<sub>4</sub>-induced hepatic fibrosis rats through attenuation of inflammation response and amelioration of metabolic function. *Food Chem Toxicol.* 2013;52:69-75.
27. Lin X, et al. Protective effect of Fufang-Liu-Yue-Qing, a traditional Chinese herbal formula, on CCl<sub>4</sub> induced liver fibrosis in rats. *J Ethnopharmacol.* 2012;142:548-556.
28. Seakins A and Robinson DS. The effect of the administration of carbon tetrachloride on the formation of plasma lipoproteins in the rat. *Biochem J.* 1963;86:401-407.