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Antioxidant activity and hepatoprotective effect of a polysaccharide from Bei Chaihu (*Bupleurum chinense* DC)

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ABSTRACT

Chinese medicine plays a pivotal role in hepatoprotective treatment. In the present study, a water-soluble polysaccharide fraction (WBCP) was fractioned from the roots of *Bupleurum chinense* and purified by DEAE-cellulose and Surperdex 200 HR chromatography. The physicochemical properties, antioxidative and hepatoprotective activities of WBCP were evaluated in a rat model of hepatic injury caused by D-galactosamine (GalN). Hepatoprotective effect was evaluated by measuring aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities in the plasma of mice. Antioxidant activity was evaluated by measuring biochemical parameters in the mouse liver homogenate, such as glutathione reductase (GR), γ-glutamylcysteine synthetase (GCS), glutathione S-transferase (GST) and superoxide dismutase (SOD) activities, as well as glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) levels. The results showed the oral administration of WBCP could significantly reduce the activity of AST, ALT, ALP and LDH, indicating that WBCP possesses hepatoprotective activity. Furthermore, there was general a statistically significant increase in the activities of GSH, GR, GCS, GST and SOD, and a loss in TBARS in the liver of WBCP-treated group compared with the control group. In addition, the elevated levels of pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) in the serum of the experimental animals was significantly returned by WBCP treatment at the dose of 400 mg/kg. These results clearly demonstrated that WBCP possess promising hepatoprotective effects against GalN-induced liver damage, which may be mediated through augmentation of antioxidant defenses.

1. Introduction

Roots of medical plants are important resources of interesting bioactive molecules, many of which have been reported to possess various biological functions (Sivakumar, 2006). *Bupleurum chinense* DC, called "Bei Chaihu", is a well-known traditional Chinese medicine distributed in Neimeng, Liaoning and Hebei province of north China, which literally means "kindling of the barbarians". As described in the Chinese pharmacopoeia, the dried root of *B. chinense* is one of the most important plant medicines in China and belongs to the plant family *Bupleurum* spp., (Ashour and Wink, 2011; Ikegami, Sumino, Fujii, Akiba, & Satoh, 2006). *B. chinense* was first mentioned in the Treatise on Cold Induced Febrile Disease, an ancient medical book, as a principal ingredient of *Xiao Chai Hu Tang*, which is an ancient Chinese medicinal formula, from the 1st century BC (Chen et al., 2009; Nishimura, Uemura, Iwamoto, & Naora, 2010). *B. chinense* possesses many pharmacological functions, such as balancing different organs and energies within the body, strengthening the action of the digestive tract, improving liver and circulatory system function, and relieving liver tension (Hsu, Huang, Tsay, Chang, & Lee, 2006). Therefore, it is also used as a popular tonic herb in China.

In the past decade, more and more researches were focused on *B. chinense*, and found that the saikosaponin, flavonoid and essential oil possessed several proven pharmacologic activities, including hepato-protective, antipyretic, mild sedative, analgesic, anti-fibrotic, antitumor and promotion of liver regeneration (Kuang, Sun, Yang, Xia, & Feng, 2009; Li, Song, Li, Chen, & Bi, 2005; Sun, 2006; Tan, Cai, Hu, & Ni, 2008). Therefore, in many Asian countries, *B. chinense* has been widely prescribed to outpatients for treating chronic liver diseases. However, there is relatively little information pertaining to isolation, purification, and hepatoprotective activity determination of water-soluble polysaccharide from *B. chinense*. Therefore, in order to fully develop the medical plant resources and extend the potential use of *B. chinense*, we specifically focused on elucidating the isolation and characterization of water-soluble polysaccharide from *B. chinense* and testing its hepatoprotective effect in a rat model of hepatic injury caused by GalN. Ultimately, the putative mechanism underlying the hepatoprotective effects was evaluated.

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2. Materials and methods

2.1. Materials and chemicals

The roots of *B. chinense* was purchased from local medicinal herb market, and identified according to the identification standard of Pharmacopeia of the People's Republic of China.

GalN, Dimethyl sulfoxide (DMSO), T-series dextrans, DEAE-cellulose, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). Surperdex 200 HR column was purchased from Amersham (Sweden). All other chemical reagents used were analytical grade.

2.2. Isolation and purification of polysaccharide fractions

After the roots of *B. chinense* were cleaned, dried, and ground, the powders were extracted with hot water for three times. The whole extract was filtered, concentrated and centrifuged, and then the supernatant was treated with 3 volumes of ethanol at 4 °C overnight. The crude polysaccharides precipitated by ethanol were washed with dehydrated alcohol and diethyl ether in turn, and collected by centrifugation, and then dried under reduce pressure. The sample dissolved in distilled water, was frozen at −20 °C, and thawed at room temperature and centrifuged to remove insoluble materials. The supernatant was deproteinated by a combination of proteinase and Sevag method (Staub, 1965), and then crude water-soluble *B. chinense* roots polysaccharides (cWBCP) was obtained.

The cWBCP was further purified on a DEAE–cellulose column, eluted successively with distilled water and 0.5 M NaCl. Fraction was collected and monitored with the phenol–sulfuric acid method. One main fraction was collected, dialyzed, lyophilized, and was further purified with Surperdex 200 HR column eluted with 0.15 M NaCl. The main fraction, codes as WBCP, was collected, dialyzed and lyophilized for further analysis.

2.3. Physicochemical property of polysaccharide fractions

Molecular weight of WBCP was determined by high performance liquid chromatography (HPLC). Total carbohydrate content of WBCP was determined by phenol-sulfuric acid colorimetric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein content was quantified according to the Bradford's method (Bradford, 1976). Total uronic acid content was measured by *m*-hydroxydiphenyl method (Filisetti-Cozzi & Carpita, 1991).

2.4. Experimental animals

Male Wistar rats weighing 200 ± 10 g, purchased from animal experimental center of the Fourth Military Medical University were used for animal experiment. The Rats were kept under standardized conditions at a temperature of 22–24°C, and 20% humidity with a 12 h light/dark cycle, and they had free access to standard rodent diet and water ad libitum. They were allowed to acclimatize for a week before the experiments were started, and they were fasted for 24 h before the experiment. Animal experiments were conducted under principles in good laboratory animal care, and approved by ethical committee for laboratory animals' care and use of the Fourth Military Medical University.

2.5. GalN-induced liver injury model in rats

Liver injury was induced by intraperitoneal injection with GalN at the dose of 400 mg/kg dissolved in saline (Watanabe et al., 2006). Blood was collected from abdominal aortas 24 h after the final GalN injection.

2.6. Experimental design

Fifty rats were randomly divided into five groups (ten mice in each group): normal (normal rats received the vehicle); negative control (GalN-treated rats received the vehicle); WBCP (GalN-treated rats received WBCP at 100, 200 and 400 mg/kg, respectively by intragastric administration for 2 consecutive weeks). All these treatments were given orally for 14 days. On the last day of the treatment, rats were fasted 7 h, and anesthetized by anesthetic ether. Then the blood was collected from the abdominal artery and kept for 30 min at 4 °C. Serum was separated by centrifugation at 2500 rpm for 15 min at 4 °C and used for test the biochemical estimations, namely AST, ALT, ALP, and LDH enzyme activity, as well as TNF-α level. The dissected livers were washed with PBS (pH 7.4) and homogenized with 0.1 M PBS (pH 7.4). Cytosolic fractions were separated from homogenates by differential centrifugation, and further used for measurement of GSH, GST, GR, GCS, and SOD activities and TBARS production.

2.7. Measurement of Serum AST, ALT ALP, LDH, TBARS and TNF-α

Serum AST, ALT, ALP and LDH were measured by assay kits from Shanhai Shensuoyoufu Bioengineering and Clinical Reagent Company. Lipid peroxidation was measured with TBARS as a marker of lipid peroxidation using TBARS assay kit from Cayman Chemical Company. TNF-α level in serum was determined by enzymelinked immunosorbent assay kit (ELISA, Cayman Chemicals, USA), according to the manufacturer's instruction.

2.8. Measurement of Hepatic GSH, GST, GR, GCS, and SOD

Hepatic GSH and activity of GST, GR, GCS and SOD were determined by assay kits from Shanhai Shensuoyoufu Bioengineering and Clinical Reagent Company.

2.9. Behavioural and acute toxicity study

Male Wistar rats were randomly divided into seven groups of six animals each. After fasted overnight, the control group received saline and the other groups received 100, 200, 400, 800, 1600, and 3200 mg/kg of polysaccharide fraction WBCP respectively. The animals were observed continuously at the first 24 h for any gross behavioural changes and toxic symptoms and 48 h for mortality rate.

2.10. Statistical analysis

Results were expressed as mean ± SD and statistical analysis was performed using ANOVA, to determine the significant differences between the groups, followed by Student Newman–Keul's test. $P < 0.05$ implied significance.

3. Results and discussion

3.1. Isolation and purification of polysaccharides from B. chinense

The crude polysaccharide from the roots of *B. chinense* was extracted by hot water and ethanol precipitation with a yield of 11.5%. After deproteinated by a combination of proteinase and Sevag method, the crude polysaccharide sample was purified by DEAE-cellulose column eluted with de-ionized water and 0.5 M NaCl, one main fraction was further purified by Surperdex 200 HR column eluted with 0.15 M NaCl. The main fraction (WBCP) was isolated for further analysis of physicochemical properties and hepatoprotective activities.

Table 1
Physicochemical properties of WBCP from *B. chinense*.

Sample	M_w (Da)	Total sugar (%)	Protein (%)	Uronic acid (%)
WBCP	94,000	95.6	nd	21.4

3.2. Physicochemical properties and chemical compositions

The contents of total sugar, protein and uronic acid, and molecular weight of WBCP are summarized in Table 1. WBCP appeared as a white powder, and had a negative response to Bradford assay. No absorption at either 280 or 260 nm was detected by UV spectrophotometer. The above results showed the absence of protein and nucleic acid in WBCP. HPLC profile indicated that WBCP had a single and symmetrically sharp peak, revealing that WBCP was a homogeneous polysaccharide, and the average molecular weight of WBCP was 94 kDa. Phenol–sulfuric acid assay showed WBCP contained 95.6% carbohydrate, and 21.4% uronic acid evaluated by m-hydroxydiphenyl method.

3.3. Protection effects against hepatic injury in rats

3.3.1. Hepatoprotective effect of WBCP on GalN-induced liver injury

GalN is a hepatotoxicant and an important inducer in hepatic injury model (Lian et al., 2010). This hepatotoxicity causes the inhibition of RNA and protein synthesis via loss of uridine nucleotides and accumulation of UDP hexosamines in hepatocytes (Decker and Keppler, 1974). In addition, GalN can increase hepatic sensitivity to TNF-α, which contributes to the hepatic injury during inflammation (Endo et al., 1999). Aminotransferase, which mediates the catalysis of amino transfer reactions, is a marker for clinical diagnosis of liver injury (Amacher, 1998). Therefore, Serum AST, ALT and TNF-α in the blood are prognostic markers in liver disease.

In order to determine the hepatoprotective effect of *B. chinense* polysaccharide, rats were treated with different concentration of WBCP after administration of GalN i.p. to mice. As shown in Table 2, GalN significantly increased serum AST and ALT activities compared with the normal control group, and WBCP could attenuate this increase in a dose-dependent manner. When treated with WBCP, AST activity was suppressed by 11.9%, 33.4% and 40.3% at the dose of 100, 200 and 400 mg/kg, respectively, and ALT activity by 8.0%, 19.4% and 30.6%, respectively.

Besides GalN can also increase serum activities of ALP and LDH. ALP as a hydrolase enzyme is responsible for removing phosphate groups from nucleotides and proteins, which is produced primarily in the liver and brain, and is a marker of hepatic function. LDH is a general indicator of acute or chronic hepatic damage (Han et al., 2006). As shown in Table 2, WBCP can significantly reduce the activity of GalN-induced ALP and LDH. When treated with WBCP (100, 200 and 400 mg/kg), ALP activity was suppressed by 8.3%, 18.3 and 27.9%, respectively, and LDH activity by 14.9%, 26.9% and 37.9%, respectively.

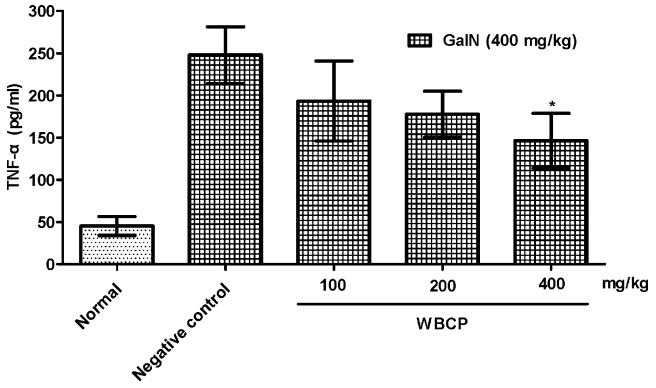

Fig. 1. Hepatoprotective effect of WBCP on serum TNF-α level in GalN-induced rats. Values are expressed as mean ± SD ($n = 10$); * $p < 0.05$, ** $p < 0.01$ vs. negative control.

TNF-α is believed to be a major endogenous inflammatory mediator of hepatotoxicity in several experimental liver damages through its direct cytotoxicity, nitric oxide production and the triggering of an inflammatory cascade (Shin et al., 2011; Tiegs, 1994). The production of TNF-α is one of the earliest events in the hepatic inflammatory response, which induces cytotoxicity, hepatocyte apoptosis and necrosis. Therefore, TNF-α is considered to be an important target in research to discover hepatoprotective agents. Fig. 1 shows the levels of pro-inflammatory cytokine TNF-α in the serum of the experimental animals. In accordance with this report, our results demonstrated that a significant elevation in the level of TNF-α was observed in the serum of GalN-treated rats, indicating the role of this cytokine in GalN-induced hepatotoxicity. When the rat models treated with WBCP at the dose of 400 mg/kg, the elevated level of TNF-α was significantly reduced. Our findings revealed that polysaccharide fraction WBCP treatment could alleviate the progression of the inflammatory mediator TNF-α in GalN-induced liver injury rats.

3.3.2. Antioxidant effect of WBCP on GalN-induced liver injury

TBARS is usually regarded as a marker of lipid peroxidation (Lovell, Ehmann, Butler, & Markesbery, 1995). TBARS levels were significantly promoted compared with normal control group when treated with GalN, whereas WBCP administration to mice significantly reduced these levels at the dose of 200 and 400 mg/kg (Fig. 2), indicating that the polysaccharide sample WBCP inhibited hepatic lipid peroxidation caused by GalN. GSH is a tripeptide that contains an unusual peptide linkage with marked antioxidant activity, preventing damage to important cellular components caused by reactive oxygen species (ROS) such as free radicals and peroxides (Franco, Schoneveld, Pappa, & Panayiotidis, 2007). As shown in Table 3, GalN can significantly reduced GSH levels in rat serum, and these changes were greatly restored by administration of WBCP at three doses of 100, 200 and 400 mg/kg. ROS is a key factor for GalN-induced hepatic damage. ROS leads to oxidation of cellular lipids and influences the antioxidant defense system, including reducing GSH levels. The above results showed that WBCP inhibited the

Table 2
Hepatoprotective effect of WBCP on liver injury induced by GalN.

Group	Dose (mg/kg)	AST (μmol/mg protein)	ALT (μmol/mg protein)	ALP (μmol/mg protein)	LDH (μmol/mg protein)
Normal		107.44 ± 9.25	74.41 ± 7.44	501.23 ± 69.42	710.61 ± 69.72
Control		932.23 ± 86.46	635.21 ± 79.32	821.59 ± 110.55	1929.45 ± 242.46
WBCP	100	821.34 ± 77.52	584.44 ± 95.24	753.43 ± 99.19	1641.48 ± 196.42
	200	621.03 ± 92.42*	512.74 ± 63.48*	671.56 ± 90.26*	1411.53 ± 241.28*
	400	556.22 ± 86.42**	441.06 ± 84.41**	592.46 ± 94.99**	1197.46 ± 243.98**

Values are expressed as mean ± SD ($n = 10$).
* $p < 0.05$.
** $p < 0.01$ vs. control.

Table 3
Antioxidant effect of WBCP on liver injury induced by GalN.

Group	Dose (mg/kg)	GSH (μmol/mg protein)	GR (μmol/mg protein)	GCS (μmol/mg protein)	GST (μmol/mg protein)	SOD (μmol/mg protein)
Normal		8.12 ± 0.88	51.34 ± 4.33	23.25 ± 5.02	271.45 ± 44.21	12.91 ± 1.24
Control		3.33 ± 0.65	31.32 ± 7.44	7.34 ± 1.93	136.24 ± 26.77	4.44 ± 0.65
WBCP	100	4.39 ± 1.13	38.12 ± 8.98	9.38 ± 2.19	151.83 ± 35.29	5.41 ± 0.71
	200	5.14 ± 0.88*	42.67 ± 8.23*	10.53 ± 2.71*	168.32 ± 39.41	5.79 ± 1.31*
	400	5.79 ± 1.16**	44.67 ± 7.76**	13.02 ± 3.01*	191.32 ± 43.21*	6.53 ± 0.81*

Values are expressed as mean ± SD ($n = 10$).
* $p < 0.05$.
** $p < 0.01$ vs. control.

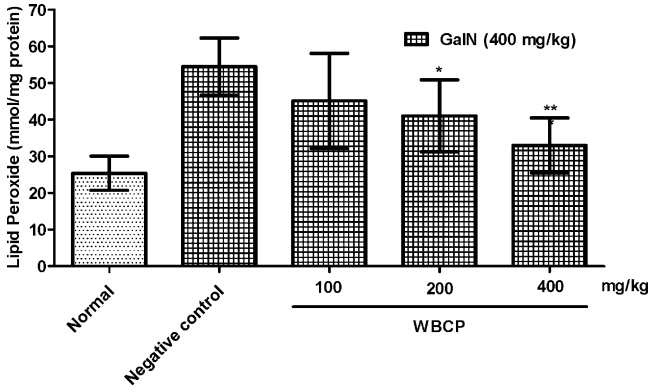

Fig. 2. Effect of WBCP on hepatic lipid peroxidation in GalN-induced liver injury. Values are expressed as mean ± SD ($n = 10$); *$p < 0.05$, **$p < 0.01$ vs. negative control.

increase of lipid peroxidation and reversed the decrease of GSH levels by GalN-induced.

The inhibition of glutathione synthesis contributed to the reduction of hepatocyte GSH content after GalN treatment, either through decreased activity of glutathione synthesizing enzymes or by increased activity of glutathione breakdown enzymes (Yoo, Nam, Kim, Choi, & Park, 2008). Oral administration of WBCP can reverse the decrease of GR and GCS activity caused by GalN treatment in rats (Table 3), thus WBCP can activate GR and GCS enzymes to prevent the reduction of hepatic GSH content induced by GalN-induced hepatic damage. Moreover, GalN significantly decreased GST and SOD levels, and then treatment with WBCP can increase GST activity by 40.4% and SOD activity by 47.1% at 400 mg/kg, respectively.

Therefore, the above results indicated the polysaccharide fraction of *B. chinense* may be a main hepatoprotective component. WBCP protects against GalN-induced hepatic injury by antioxidant mechanisms.

3.4. Acute toxicity

For the acute toxicity studies, no death of rat was recorded either in the control or in the treated groups. The animals did not exhibit any gross behavioural changes within 24 h at six doses, and no mortality happened for 48 h. The results showed that the polysaccharide fraction from *B. chinense* has no toxicity to experimental rats.

4. Conclusion

B. chinense, as important traditional Chinese medicine, has been used for treating chronic liver diseases over thousand years (Wang, Kong, Wang, Lien, & Lien, 2007). However, the specific active chemical compositions and the possible mechanism have been still unclear. Therefore, in the present investigation, a water-soluble polysaccharide WBCP was extracted from the roots of *B. chinense*, and then hepatoprotective activities of WBCP were evaluated in a rat model of hepatic injury caused by GalN.

Physicochemical assays showed WBCP was a homogeneous polysaccharide with average molecular weight of 94 kDa, contained 95.6% carbohydrate and 21.4% uronic acid. No protein and nucleic acid was detected in the sample. Further, the hepatoprotective activities of WBCP were evaluated in a rat model of hepatic injury caused by D-GalN. Oral administration of WBCP could significantly reduce the activity of AST, ALT, ALP and LDH, and TNF-α level in the serum of GalN-treated rats, indicating that WBCP possessed hepatoprotective activity. WBCP also increased activity levels of GSH, GR, GCS, GST and SOD, suggesting that WBCP exhibited hepatoprotective activity maybe due to antioxidant mechanism. These results suggested WBCP could be considered as a potential candidate for developing a new hepatoprotective agent.

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