Hepatoprotective Effects of the Polysaccharide Isolated from *Tarphochlamys affinis* (Acanthaceae) against CCl₄-Induced Hepatic Injury

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This study was designed to investigate the protective effects of the polysaccharide isolated from *Tarphochlamys affinis* (PTA) against CCl₄-induced hepatotoxicity in rats. Liver injury was induced in rats by the administration of CCl₄ twice a week for 2 weeks. During the experiment, the model group received CCl₄ only; the treatment groups received various drugs plus CCl₄, whereas the normal control group received an equal volume of saline. Compared with the CCl₄ group, PTA significantly decreased the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in the serum and increased the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) in the liver. Moreover, the content of hepatic malondialdehyde (MDA) was reduced. Histological findings also confirmed the antihepatotoxic characterisation. In addition, PTA significantly inhibited the proinflammatory mediators, such as prostaglandin E₂ (PGE₂), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and myeloperoxidase (MPO). Further investigation showed that the inhibitory effect of PTA on the pro-inflammatory cytokines was associated with the down-regulation of nuclear factor-kappa B (NF-κB). In brief, our results show that the protective effect of PTA against CCl₄-induced hepatic injury may rely on its ability to reduce oxidative stress and suppress inflammatory responses.

Key words  *Tarphochlamys affinis* (Acanthaceae); polysaccharide; hepatoprotective effect; carbon tetrachloride

In recent years, “oxidative stress” and its adverse effects on human health has become a subject of considerable interest of research. It is a well-documented fact that exposure of organisms to the exogenous and endogenous factors generates a wide range of reactive oxygen species (ROS), resulting in the homeostatic imbalance.¹ ² Production of free radicals exceeded, such as superoxide, hydroxyl radicals, hydrogen peroxide and nitric oxide (commonly designated as ROS), was found to play multiple important roles in tissue damage and the loss of function.³ Development in the biomedicine points to the involvement of the free radicals in many diseases. The free radicals attack the unsaturated fatty acids in the biomembranes, yielding the membrane lipid peroxidation, the decrease in membrane fluidity, the loss of enzymes and the damage to membrane proteins and leading to the cell inactiva-tion.⁴ The harmful action of the free radicals can, however, be blocked by the antioxidant substances, which scavenge the free radicals and detoxify the organism. Many studies have shown that natural polysaccharide, which were found largely in fruits and vegetables, have been confirmed to play an important part as free radical scavengers in the prevention of oxidative damage in living organism and can be exploited as novel potential antioxidants, and the effects have something to do with their chemical properties and architectural characteristics.⁵ Therefore, discovery and assessment of natural polysaccharide as new safe compounds for functional foods or medicines have become a hot research field.

*Tarphochlamys affinis* (Acanthaceae) has been used in Chinese traditional medicine with a long history for the remedy of tumors, acute or chronic hepatitis and jaundice. In our previous studies, the ethanol extract of the root has been shown of anti-virus effect on hepatitis B virus.⁶ Further, the petroleum, chloroform, ethyl acetate and n-butyl alcohol extracts have been reported to improve the immune function in immunosuppressed mice induced by cyclophosphamide.⁷ Besides, the polysaccharide from the root has also been demonstrated of antioxidant property in vitro.⁸ However, there is relatively little information pertaining to hepatoprotective activity determination of polysaccharide from *Tarphochlamys affinis*. Therefore, in order to fully develop the medical plant resources and extend the potential use of *Tarphochlamys affinis*, we specifically focused on elucidating the isolation and characterization of polysaccharide from *Tarphochlamys affinis* and testing its hepatoprotective effect in a rat model of hepatic injury caused by CCl₄. Ultimately, the putative mechanism underlying the hepatoprotective effects was evaluated. Silymarin, a polyphenolic flavonoid isolated from milk thistle (*Silybum marianum*), is used clinically in Europe and Asia for the treatment of liver diseases. Various studies indicated that silymarin exhibits strong antioxidative activity.⁹ ¹⁰ In this study, silymarin was used as positive control.

MATERIALS AND METHODS

Chemicals  Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), superoxide dismutase (SOD), glutathione peroxidase (GPx), malondialdehyde (MDA) kits were purchased from Nanjing Jianchen Biotechnology Co., Ltd., China. Enzyme-linked immunosorbent assay (ELISA) kits for interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and prostaglandin E₂ (PGE₂) were purchased from Sino-American Biological Technology Co., Ltd., France, respectively. Silymarin was purchased from Sigma-Aldrich Corporation, St. Louis, MO, USA.

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Preparation of the Polysaccharide Isolated from *Tarphochlamys affinis* (PTA)  
*Tarphochlamys affinis* (Acanthaceae) was purchased from Nanning Qianjinzi Chinese Pharmaceutical Co., Ltd. (Nanning, China). Voucher specimen (LYQ2010070301) were identified by Professor Q. F. Huang in Department of Pharmacognosy, The First Affiliated Hospital of Guangxi University of Chinese Medicine and deposited in the herbarium of Department of Pharmacology of Guangxi Medical University.

The polysaccharide was prepared according to the previous study\(^1\) with slight modification. The dry and powdered *Tarphochlamys affinis* (1000 g) was extracted with 10000 mL water for 2 h at 95–100°C 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 precipitate was separated by filtration, washed exhaustively with 95% ethanol, and then dissolved in deionized water and dialyzed using cellulose sacks (8000–10000 Da, Sigma) against flowing water for 48 h. The non-dialyzed portion was precipitated again. The resulting precipitate was filtered and washed exhaustively with anhydrous ethanol, acetone and anhydrous ether subsequently, and then PTA was obtained.

In addition, the total of carbohydrate content in PTA was determined by phenol–sulfuric acid colorimetric method with glucose as a standard.\(^2\) According to the ordinary procedure, absorbance at 490 nm of five calibration solutions of glucose (0.406, 0.812, 1.218, 1.624, 2.030 mg/mL) were determined, and the standard curve was drawn with absorbance as ordinate and concentration (mg/mL) as abscissa, and the regression equation was obtained. The amount of carbohydrates present in PTA was determined by comparison with a calibration curve using a spectrophotometer. In brief, 50 mg PTA was put into volumetric flask (25 mL) to get stock solution once it was completely dissolved in ultrapure water, and the working solution was prepared by diluting the stock solution to the appropriate concentrations. Fifty microliters of working solution was spiked with 500 µL of 4% phenol, followed by 2.5 mL sulfuric acid. Under the catalysis of sulfuric acid, PTA was converted to monosaccharides, then to derivants of pyromucic acid (EDTA), pH 7.4) to give a 10% (w/v) liver homogenate. The homogenates were then centrifuged at 1000 rpm for 10 min at 4°C and the supernatants were used immediately after the determination of absorbance of the orange-yellow chemical compounds.

**Experimental Animals**  
Male Sprague-Dawley (SD) rats (200±10 g) were obtained from the Experimental Animal Centre of Guangxi Medical University [SCXXG 2003-0003] and were allowed to acclimate in quarantine for a week prior to experimentation. The research was conducted according to protocols approved by our institutional ethical committee. The animals were handled under the standard laboratory conditions of a 12-h light/dark cycle in a room with controllable temperature and humidity. Food and water were available *ad libitum*.

**Experimental Design** The animals were randomly divided into six groups of 12 rats per group. Group I served as a normal control and was given peanut oil (1 mL/kg) twice a week for a period of 2 weeks. To induce hepatotoxicity (*in vivo*), the animals in Groups II–VI received 1 mL/kg body weight of CCl₄ (20% CCl₄ in peanut oil) orally twice a week for 2 weeks. Group II served as the CCl₄-treated model group. In addition to CCl₄, Group III was given silymarin (50 mg/kg) orally daily for a period of 2 weeks, which served as a positive control. In addition to CCl₄ administration, Groups IV–VI received PTA orally (70, 140, 280 mg/kg, representative of low, medium and high dosage, respectively) daily for 2 weeks. At the end of the experiment, the animals were sacrificed by cervical dislocation. Serum samples were collected in heparinized tubes (50 U/mL). Liver samples were dissected out and washed immediately with ice-cold saline to remove as much blood as possible. One part of each liver sample was immediately stored at −80°C until analysis, and another part was excised and fixed in 10% formalin solution for histopathologic analysis.

**Estimation of the Levels of Serum Marker Enzymes and Hepatic PGE₂**  
To assess the liver injury, activities of the serum AST, ALT and ALP were assayed using kits obtained from Nanjing Jianchen Bioengineering Institute, Nanjing, China. The hepatic PGE₂ level was measured using commercially available ELISA kit (R&D Systems, U.S.A., and Diaclone Research, France, respectively).

**Assay of Plasma IL-6, TNF-α and Liver Myeloperoxidase Activities** Plasma IL-6 and TNF-α were assayed by sandwich enzyme linked immunosorbent assay method following the protocol provided by the manufacturers. MPO activity was measured according to the method of Yoshida et al.\(^3\) Tissue was homogenized in 50 mM phosphate buffer, pH 6.0 containing 0.5% hexadecl trimethyl ammoniumbromide. The supernatant obtained after centrifugation was mixed with 10 mM phosphate buffer (pH 6.0) and 1 mL of 1.5 mM o-dianisidine hydrochloride containing 0.2 mM H₂O₂. The change in absorbance at 450 nm of each sample was recorded. MPO activity was expressed as µmol of the oxidized product formed/min/mg protein using the extinction coefficient of 10062 µM⁻¹ cm⁻¹. Protein content was determined using bovine serum albumin as standard.

**Measurement of MDA, SOD and GPxs in Liver Homogenate** Liver samples were homogenized in Tris–HCl buffer (5 mM/L containing 2 mM/L ethylenediaminetetraacetic acid (EDTA), pH 7.4) to give a 10% (w/v) liver homogenate. The homogenates were then centrifuged at 1000 rpm for 10 min at 4°C and the supernatants were used immediately for the determination of antioxidant status. Activities of antioxidant defense enzymes, SOD and GPxs, as well as the level of MDA, as an index of the extent of lipid peroxidation in liver tissue, were determined following the instructions on the kit. In brief, MDA content was determined by the thiobarbituric acid method. The assay for total SOD was based on its ability to inhibit the oxidation of oxyamine by the xanthine–xanthine oxidase system, while GPxs was measured by the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) method. All samples were assayed in triplicates. The content of MDA was expressed as nanomole, while SOD and GPxs activities were expressed as units per milligram protein. Protein content of the homogenates was determined using a standard commercial kit provided by Jiancheng Institute of Biotechnology (Nanjing, China).

**Histological Analysis of Liver** The liver samples were sectioned and stained with haematoxylin–eosin (H&E) and subsequently examined under a light microscope (IX51,
Olympus, Japan) for general histopathology examination. The extent of hepatic damage was evaluated on H&E slides. The histological changes were scored according to the following criteria: 0, absent; 1, mild; 2, moderate; and 3, severe.14)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of Hepatic Inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2) Total RNA was extracted and first strand cDNA was synthesized by reverse transcription of the total RNA using the oligo(dT)12-18 primer and SuperScript™ II RNaseH–Reverse Transcriptase (Invitrogen TechLine™, Carlsbad, CA, U.S.A.). The PCR primers were designed according to the sequence in GenBank and were as follows: for iNOS, sense: 5’-AAG CTT GAT GTG ACA TCG ACC CGT-3’, antisense: 5’-GCA TCT GTG AGC CAG CGT ACC GGG-3’, 598 bp; COX-2, sense: 5’-ACT CAC TCG TGT TGA GTC ATT C-3’, antisense: 5’-TTT GAT TAG TAC TGT AGG GTT AAT G-3’, 583 bp; β-actin, sense: 5’-TGT CAT CCT CGG ATG CGT-3’, antisense: 5’-TGG AAT CTT GTG GCA TCC ATG AAA-3’, 348 bp. The PCR reaction was carried out in a 20 µL reaction volume with a diluted cDNA sample. The amplification cycling conditions were as follows: for iNOS, 35 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s; for COX-2 and β-actin, 35 cycles and 25 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, respectively. After PCR, 10 µL samples of the PCR products were electrophoresed through 1.5% agarose gel, stained with ethidium bromide, and visualized by UV illumination. Semiquantitative analysis of the intensity of each PCR product was performed using SLB MylImager (UVP Inc., Upland, CA, U.S.A.) and ImageQuant™ TL (Amersham Biosciences/GE Healthcare, U.S.A.).

Assay for Nuclear Factor-κB (NF-κB) NF-κB p65 DNA-binding activity in nuclear extracts of hepatic tissue samples was evaluated to measure the degree of NF-κB activation. NF-κB p65 DNA-binding activity was detected according to the previous study.15) The assay is based on the sandwich enzyme linked-immunosorbent assay (ELISA). The analysis was performed according to the manufacturer’s protocol for a commercial kit (NF-κB p65 Colourimetric Transcription Factor Assay, Invitrogen Corporation, Carlsbad, CA, U.S.A.). This method combines the principle of the electrophoretic mobility shift assay with the 96-well-based enzyme linked-immunosorbent assay (ELISA) method. The extraction yield of PTA can reach approximately 0.83% (w/w) of the dried herb. The phenol–H2SO4 assay for total carbohydrate content showed that PTA contained approximately 71.8% (w/w) of polysaccharide. The linearity of the method was assessed by preparing five calibration solutions of glucose (n=5), respectively. As a consequence, a good linearity was obtained by regression analysis between A (absorbency) and C (glucose concentration, mg/mL) and regression equation was as follows: A=0.3032C+0.0325 (R²=0.9988) for glucose at the concentration of 0.406–2.030 mg/mL.

Effects of PTA on Serum ALT, AST and ALP The effects of PTA treatment on the CCl4-induced modification in the serum ALT, AST and ALP levels were shown in Fig. 1. Hepatotoxicity induced by CCl4 in rats, as indicated by an increase in the serum ALT, AST and ALP activities after CCl4 administration. In contrast, the animals treated with PTA exhibited a significant decrease in the activities of these serum marker enzymes. Silymarin administration also reversed the alterations of the ALT, AST and ALP levels compared with the normal control group.

Histopathologic Examination Examination of liver histo-
tology revealed that CCl₄ could induce histological changes including increased degeneration, necrosis, hepatitis and portal triaditis. All rats except those in the control group exhibited the ballooning degeneration in the centrolobular zone and the necrosis of hepatocytes. The CCl₄-induced damage suffered more severely than other groups treated with silymarin or PTA. It seems likely that CCl₄ administration caused oxidative stress in liver via the generation of free radicals whereas PTA ameliorates the liver injuries by scavenging of free radicals, which is further confirmed by the reduced amount of histopathological injury (Fig. 4).

**Effects of PTA on iNOS and COX-2 mRNA Expression**

The levels of iNOS and COX-2 mRNA in the CCl₄ group were higher than that of the normal control level, respectively. In contrast, the increases in iNOS and COX-2 mRNA expression were significantly suppressed by PTA (Fig. 5).

**Effect of PTA on Liver PGE₂ Level**

PGE₂ is important prostanoid that is involved in hepatocyte damage induced by CCl₄. The level of PGE₂ was low in the normal control animals. However, the level of the prostanoid increased by approximately 3.0-fold in the CCl₄-treated animals after the CCl₄ treatment. The increase was reduced in both the PTA- and the silymarin-supplemented groups (Fig. 6).

**Effects of PTA on Plasma IL-6 and TNF-α Levels and Liver Myeloperoxidase Activity**

The levels of IL-6 and...
TNF-α, as well as the activity of MPO in the model group were significantly higher compared to those in the normal control group. Up-regulation was markedly inhibited after treated with PTA (Fig. 7).

**Effect of PTA on the Translocation of NF-κB** NF-κB plays a critical role in chronic inflammatory diseases, and its activation is essential for cytokine production. In this study, we have observed remarkable activation of NF-κB (NF-κB DNA binding activity) in the model control group compared with the normal control group. Treatment with PTA was found to significantly restrict the NF-κB activation in Groups V and VI (Fig. 8).

**DISCUSSION**

The polysaccharide is believed to be one of the major active ingredients of medicinal plants. In the past several decades, the polysaccharides isolated from Chinese herbs have been reported to possess activity in stimulating the function of phagocytes, inhibiting the tumor development, protecting of liver function, etc. The polysaccharides were considered to play an important role as radical scavenger for the prevention of oxidative damage to the living systems. In our pilot study, the extract of PTA significantly inhibited the generation of MDA, the hemolysis of RBC, the swelling of mitochondria and the production of OH radical by PTA. The results indicated that PTA might has a potential protective effect against CCl4-induced liver injury.

It is well recognized that liver injury induced by CCl4 resulted from free radicals which cause lipid peroxidation, leading to hepatic cell damage. MDA is one of the end-products of polyunsaturated fatty acid peroxidation and its tissue level can reflect the extent of lipid peroxidation in hepatocytes. In the present study, the increase in MDA level observed in liver homogenate of CCl4-intoxicated rats was almost completely prevented by treatment with the highest dose of PTA, indicating its ability to break the chain reaction of lipid peroxidation.

In order to more clarify the mechanisms of hepatoprotective activity of PTA, the effect on hepatic antioxidant defense system was explored. SOD and GPx are two antioxidant enzymes which play a critical role in the cellular defense against the deleterious action of ROS and cellular products of free radical chain reactions. While SOD catalyzes the conversion of...
superoxide free radical to less toxic hydrogen peroxide, GPx catalyzes the breakdown of hydrogen peroxide into water and oxygen and can also directly detoxify lipid peroxides generated by ROS. As SOD and GPx are easily inactivated by ROS or lipid peroxides, this may explain a decrease in activities of these two enzymes observed in liver tissue of CCl₄-intoxicated rats in our study. However, our results demonstrated that SOD and GPx were appreciably elevated by PTA administration, suggesting that it could restore both enzymes and/or activate enzyme activities in CCl₄-damaged liver tissue. Moreover, these findings supported the beneficial effect of PTA in maintaining the hepatocytes integrity and function. It was conceivable that these effects might be due, at least in part, to its antioxidant activity.

As it is well known, inflammation was initiated by CCl₄-induced hepatotoxicity, with release of pro-inflammatory mediators, such as iNOS, COX-2, PGE₂, IL-6, TNF-α and MPO. Certain evidence has indicated that hepatic damage could be caused by excessive nitric oxide production through iNOS. Nitric oxide (NO) is a highly reactive oxidant that is produced through the action of NOS and plays an important role as a vasodilator and neurotransmitter and in the immunological system as a defence against tumour cells, parasites and bacteria. However, the excessive accumulation of NO would cause damage to the liver. IL-6, TNF-α and MPO another factors contributing to liver damage, are pleiotropic pro-inflammatory cytokines that are rapidly produced by macrophages in response to tissue damage, which conversely stimulates the release of cytokines from macrophages and promotes the oxidative metabolism and nitric oxide production of the phagocyte. Additional factors closely related to NO production included COX-2 activity and the subsequent generation of PGE₂. COX-2 is the rate-limiting enzyme and is responsible for the catalysis of PGE₂ from arachidonic acid. The overproduction of PGE₂ mediated by COX-2 has been linked to the development of inflammation and carcinogenesis. Consistent with the previous studies, there were significant increases in iNOS, COX-2 and PGE₂ productions as well as IL-6, TNF-α and MPO levels after CCl₄ administration in this study, confirming that an inflammatory response was elicited at the site of injury. In contrast, the expression levels of these pro-inflammatory factors were down-regulated after treatment with PTA, which may suppress the secretion and/or enhance the degradation of these proteins.

Further exploration of the underlying mechanism of PTA on the pro-inflammatory mediators showed that PTA played a crucial role in the reduction of NF-κB activation. NF-κB, a transcription factor, plays a central role in general inflammation and in tumourigenesis. The rapid phosphorylation of IκBα and its subsequent degradation following exposure of cells to external stimuli, such as carcinogens, inflammatory cytokines and reactive oxygen species, leads to increased nuclear translocation and DNA binding of NF-κB. Our results showed that CCl₄ significantly increased the NF-κB activation. However, treatment of rats with PTA suppressed the NF-κB activation significantly towards normal level. This result indicated that PTA inhibited inflammatory responses, in large part, via the down-regulation of NF-κB.

In conclusion, our study demonstrated that PTA was significantly beneficial in the prevention of CCl₄-induced liver toxicity, possibly by scavenging reactive free radicals, boosting the endogenous antioxidant system, and inhibiting pro-inflammatory cytokines via the down-regulation of NF-κB. Further research to explicate the shielding effects of PTA in CCl₄-induced liver damage is important and will provide additional evidence for the exploitation of its broader remedial usage.

Acknowledgments

The authors gratefully acknowledge financial support by the Guangxi scientific research and technology development research projects (0015048; 10124008-6; 0322024-5E), Guangxi Natural Science Foundation (No. 0731066), Guangxi Key Laboratory for Prevention and Treatment of Regional High-Incidence Diseases (KFJJ2010-22).

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