Hepatoprotective and Antioxidant Activities of the Aqueous Extract from the Rhizome of *Phragmites australis*

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The rhizome of *Phragmites australis* has long been used for the treatment of hepatitis in traditional Chinese medicine. In this study, the hepatoprotective and antioxidant activities of an aqueous extract from the rhizome of *P. australis* (AE-PA) were evaluated. The acute toxicity test in mice showed that AE-PA was nontoxic since a dose of 2000 mg/kg body weight (b.w.) did not cause toxic symptoms or mortality. The prolongation of hexobarbital-induced sleeping time by carbon tetrachloride (CCl_4) administration to mice was significantly reduced after pretreatment with AE-PA at 500 mg/kg b.w., proving the protective effect of the extract on microsomal drug-metabolizing enzyme. The oral administration of AE-PA to rats for 5 days before CCl_4 intoxication caused a significant decrease in the CCl_4-induced elevation of hepatic enzymes activities in serum, such as aspartate aminotransferase, alanine aminotransferase, and lactic acid dehydrogenase. This suggested that AE-PA had good hepatoprotective activity against CCl_4-induced liver injury, which was confirmed by pathomorphological examination of the liver. Through evaluation of hydroxyl radical and superoxide anion radical scavenging activities, respectively, it was demonstrated that AE-PA had good antioxidant activity, which possibly contributed to its hepatoprotective activity. More research is needed to study the bio-active compounds in *P. australis* and to identify the potential hepatoprotective and antioxidant agents.

Key words: Phragmites australis, Hepatoprotective, Antioxidant

Introduction

Phragmites australis (Cav.) Trin. ex Steud. is a large perennial grass found in wetlands throughout temperate and tropical regions of the world. It plays very important ecological roles in the world and has been termed the "second forest". In addition, *P. australis* also has important pharmaceutical value. Many active components have been isolated from *P. australis*, such as terpenoids, flavonoids, coumarins, acetylenes, caffeoylquinic acids, sterols, and amylase (Gao *et al.*, 2009; SATCM, 1999). The rhizoma of *P. australis* have long been used for the treatment of pulmonary diseases in traditional Chinese medicine. It was also reported that rhizoma of *P. australis* could be used to cure hepatitis (Wu, 1986). However, there are no pharmacological studies on the hepatoprotective effect of *P. australis*.

Boiling water is a classical method to extract active components from Chinese herbs and has been used in many studies (Wang *et al.*, 2005; Tang *et al.*, 2011). In the present study, this method was also used to prepare an aqueous extract from the rhizome of *P. australis* (AE-PA) whose hepatoprotective effect in rats suffering from hepatic injury induced by carbon tetrachloride (CCl_4) was evaluated. Acute toxicity and hexobarbital-induced sleeping time tests, respectively, were also carried out. The antioxidant activity of AE-PA was determined by its ability to scavenge hydroxyl radicals and superoxide anion radicals. The objective of this work was to disclose the pharmaceutical value of *P. australis* in hepatoprotection.

Material and Methods

Plant material

Rhizomes of *P. australis* were collected from Anhui, China, and identified by Prof. Hai-Liang Zhu of Nanjing University, Nanjing, China. A voucher specimen was deposited at the herbarium of Nanjing University.

Preparation of aqueous extracts

Aqueous extracts were prepared as reported by Sun and Xu (2002), with some modifications. Briefly, the material (100 g) was extracted twice with the threefold volume of water (1 L) at 100 °C for 1 h each time. The supernatants from each extraction were combined and centrifuged for 15 min at 2970 x *g*. The resulting precipitate was discarded, the supernatant was concentrated to 500 mL under vacuum, then freeze-dried to give 9 g AE-PA powder.

Chemicals

Hexobarbital, CCl_4, and silymarin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Commercial reagent kits for the determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactic acid dehydrogenase (LDH) were purchased from EHSY Company (Shanghai, China).

Animals

Male BALB mice [weighing (20 ± 2) g] and Wistar rats [weighing (180 ± 20) g] were purchased from Yangzhou University (Yangzhou, Jiangsu Province, China). The animals were maintained under a controlled 12-h light/12-h dark cycle, at (22 ± 2) °C and (65 ± 5)% relative humidity, and fed the common diet with free access to drinking water according to state standard (MHC, 1994).

Acute toxicity test

According to the OECD guidelines, 2000 mg/kg body weight (b.w.) is the maximum dose for which testing is legitimate (OECD, 1993). Ten mice were given orally 2000 mg/kg b.w. of AE-PA and were kept under observation for two weeks. The animals did not show any signs of toxicity and no mortality was observed.

Hexobarbital-induced sleeping time of mice

The experiment was performed according to the described model with some modifications (Montilla *et al.*, 1990; Adamska *et al.*, 2003). Male mice were divided into five groups of ten mice each. Group A received olive oil (5 mL/kg b.w.) and served as a control. Group B was given the same amount of olive oil and after 1 h a solution of CCl_4 in olive oil (0.5 mL/kg b.w.). Groups C – E were administered an olive oil solution of 100, 200, and 500 mg/kg b.w. AE-PA, respectively, and after 1 h a solution of CCl_4 in olive oil (0.5 mL/kg b.w.). Then, after 1 h all animals were given a saline solution of hexobarbital (60 mg/kg b.w.), and the sleeping time was recorded. All substances were administered by intraperitoneal injection.

Assessment of hepatoprotective activity

Male rats were divided into six groups of ten animals each. Group I received orally normal saline alone (50 mL/kg b.w.) and served as a blank control. Group II was given the normal diet as negative control. Groups III, IV, and V were orally treated with 100, 200, and 500 mg/kg b.w. of AE-PA, respectively, while group VI, serving as a positive control, received 100 mg/kg b.w. silymarin. Administrations by gavage were given daily for 5 d. Two h after administration of the final sample, group I was given a dose of saline (10 mL/kg b.w.) by intraperitoneal injection; and groups II–VI were given a dose of CCl_4 solution in olive oil [0.15% (v/v), 10 mL/kg b.w.] by intraperitoneal injection. Then the rats were placed on an overnight fast, orbital blood samples were collected under diethyl ether anaesthesia, and serum was obtained by centrifugation at 1970 x *g* for 10 min. The animals were sacrificed and the livers immediately excised. Samples of the livers were fixed in 10% neutral formalin, dehydrated with ethanol, embedded in paraffin, stained with haematoxylin and eosin, and examined by light microscopy. Activities of ALT, AST, and LDH in serum were analysed according to the manufacturer's procedures.

Assessment of antioxidant activity

The effect of AE-PA on hydroxyl radical scavenging was determined using the 1,10-phenanthroline method (Jin *et al.*, 1996; Liang *et al.*, 2011). The reaction mixture consisted of 1,10-phenanthroline (1 mL, 7.5 mM), phosphate-buffered saline (2 mL, 0.2 M, pH 7.4), $FeSO_4$ (1 mL, 7.5 mM), H_2O_2 (1 mL, 0.1%), and AE-PA solution (1 mL). After incubation at 37 °C for 1 h, absorbance was determined at 536 nm. Percentage of hydroxyl radical

scavenging activity was calculated using the following formula:

percentage of hydroxyl radical scavenging activity (%) =

$$\frac{A_s - A_d}{A_0 - A_d} \cdot 100\%, \quad (1)$$

where A_s is the absorbance in the presence of both AE-PA and H_2O_2, A_d is the absorbance in the presence of H_2O_2 and the absence of AE-PA, and A_0 is the absorbance in the absence of both AE-PA and H_2O_2.

Superoxide anion radical scavenging activity was evaluated using the pyrogallol method (Liang *et al.*, 2011; Marklund and Marklund, 1974). Tris-HCl buffer (4.5 mL, 50 mM, pH 8.2) and distilled water (4.2 mL) were added to freshly prepared pyrogallol solution (0.3 mL, 3 mM, dissolved in 10 mM HCl). The absorbance at 325 nm was measured at 0.5-min intervals for 4 min. Pyrogallol was replaced by HCl in the blank control. The increment of the absorbance rate in the absence and presence of AE-PA was regarded as A_1 and A_2, respectively. Percentage of superoxide anion radical scavenging activity was calculated using the following formula:

percentage of superoxide anion radical scavenging activity (%) =

$$\frac{A_1 - A_2}{A_1} \cdot 100\%. \quad (2)$$

Statistics

All data were expressed as mean ± standard deviation (S.D.). Statistical evaluation by Student's t-test was performed when only two value sets were compared using Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA, USA), and one-way analysis of variance (ANOVA) followed by Dunnett's test when the data involved three or more groups using SPSS 13.0 for windows (IBM Corporation, Armonk, NY, USA). A difference of $p < 0.05$ was considered to be significant.

Results

The acute toxicity test revealed that the aqueous extract of the *P. australis* rhizome was non-toxic, since 2000 mg/kg b.w. of AE-PA did not cause toxic symptoms or mortality.

The effect of CCl_4 and AE-PA on the hexobarbital-induced sleeping time of mice was examined. As shown in Table I, hexobarbital at a dose of 60 mg/kg b.w. caused sleep for a period (27.2 ± 3.2) min. Pretreatment of animals with CCl_4 prolonged this time to (137.6 ± 25.6) min, whereas in animals that were given AE-PA prior to CCl_4 administration, the recorded sleeping time decreased in a dose-dependent manner compared with group B.

Table II summarizes the effects of CCl_4 and AE-PA on the activities of hepatic enzymes in the serum of rats. After injection of CCl_4, serum activities of ALT, AST, and LDH in animals of group II significantly increased ($p < 0.01$) compared with group I, indicating that the liver injury model was established successfully. Pretreatment with AE-PA and silymarin resulted in a decrease of ALT, AST, and LDH activities. As shown in Table II, the hepatic enzyme activities of groups III–V were lower than those of group II. The decrease was dose-dependent, and the effect of the highest dose of AE-PA (500 mg/kg b.w.) was comparable to that of silymarin at 100 mg/kg b.w.

The effects of CCl_4, AE-PA, and silymarin on the liver histopathology of rats are presented in Fig. 1. As seen in Fig. 1A, the liver section of normal rats showed distinct hepatic cells with well preserved cytoplasm, prominent nucleus and nucleolus. While liver sections of CCl_4-treated rats showed patches of cell necrosis with inflammatory conditions (Fig. 1B), these were reduced in the AE-PA-treated groups (Figs. 1C–E). The group treated with 500 mg/kg b.w. AE-PA had only minimal inflammatory conditions with near normal liver architecture (Fig. 1E), similar to the silymarin-treated group (Fig. 1F).

Table I. Effect of CCl_4 and AE-PA on the hexobarbital-induced sleeping time of mice.

Group	CCl_4	Dose of AE-PA (mg/kg b.w.)	Sleeping time [min]
A	–	0	27.2 ± 3.2
B	+	0	137.6 ± 25.6$^{\#\#}$
C	+	100	119.2 ± 19.2
D	+	200	93.6 ± 8.7*
E	+	500	62.4 ± 7.9**

$^{\#\#}$ $p < 0.01$ significantly different in comparison with group A; * $p < 0.05$, ** $p < 0.01$ significantly different in comparison with group B.

Table II. Effect of CCl_4 and AE-PA on the activities of hepatic enzymes in the serum of rats.

Group	CCl_4	Dose of AE-PA (mg/kg b.w.)	ALT [U/L]	AST [U/L]	LDH [U/L]
I	–	0	27.2 ± 10.5	35.3 ± 12.5	20.0 ± 3.4
II	+	0	163.4 ± 26.1##	161.3 ± 28.8##	157.0 ± 36.7##
III	+	100	140.3 ± 23.6	150.1 ± 32.4	103.4 ± 21.9*
IV	+	200	98.0 ± 12.3*	69.6 ± 24.2**	80.1 ± 12.3*
V	+	500	57.6 ± 7.7**	66.0 ± 10.4**	42.9 ± 18.4**
VI	+	100 (Silymarin)	37.5 ± 30.8**	79.9 ± 27.6**	79.8 ± 24.0**

$p < 0.01$ significantly different when compared with group I; * $p < 0.05$, ** $p < 0.01$ significantly different when compared with group II.

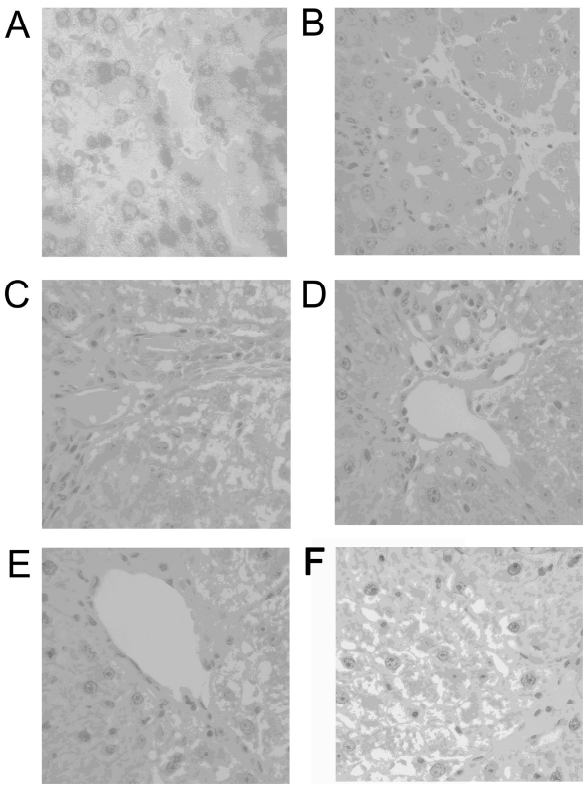

Fig. 1. Effect of CCl_4 and AE-PA on the liver histopathology of rats. Liver sections of (A) normal group; (B) CCl_4 control group; (C) CCl_4 + AE-PA group (100 mg/kg b.w.); (D) CCl_4 + AE-PA group (200 mg/kg b.w.); (E) CCl_4 + AE-PA group (500 mg/kg b.w.); (F) CCl_4 + silymarin group (100 mg/kg b.w.).

The effects of AE-PA on hydroxyl radical and superoxide anion radical scavenging activities are displayed in Fig. 2. The activities were dose-dependent. When the concentration of AE-PA was 50 mg/mL, the relative radical scavenging activity reached approximately 62%.

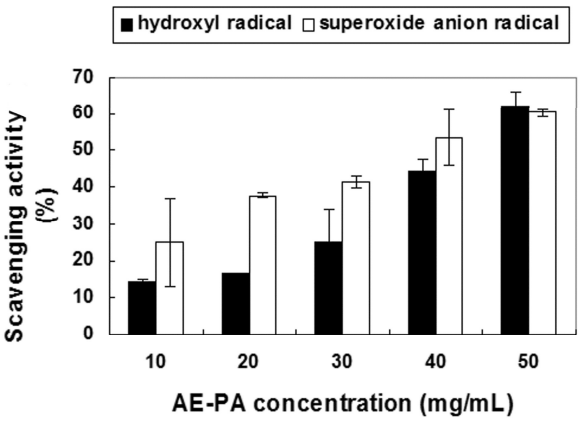

Fig. 2. Hydroxyl radical and superoxide anion radical scavenging activities of AE-PA.

Discussion

Carbon tetrachloride-induced hepatotoxicity is a widely used animal model for hepatoprotective drug screening. The hepatotoxic effects of CCl_4 are thought to result from its reductive dehalogenation to the highly reactive free radical ($^{\bullet}CCl_3$) by cytochrome P450 enzyme systems (Jia *et al.*, 2011). This radical can combine with molecular oxygen to form the trichloromethyl peroxyl radical ($^{\bullet}OOCCl_3$), which abstracts hydrogen from unsaturated lipids and initiates the process of lipid peroxidation (Williams and Burk, 1990).

In addition, products of CCl_4 biotransformation damage hepatic microsomal drug-metabolizing enzymes (MDME), which metabolize barbiturates to inactive metabolites. Thus, CCl_4 derivatives inhibiting MDME will prolong the barbiturate-induced sleeping time. Pretreatment of animals with substances which stimulate or protect MDME can reduce the prolonged sleeping time, which is a test commonly used for the screening of hepatoprotective drugs (Adamska *et*

al., 2003; Gilani and Janbaz, 1995). In our experiment, the increase of the hexobarbital-induced sleeping time caused by CCl_4 was markedly reduced when animals had been pretreated with AE-PA. At 500 mg/kg b.w. of AE-PA, the hexobarbital-induced sleeping time was decreased by approximately 55%.

Liver damage can be assessed by biochemical parameters. ALT, AST, and LDH levels are most frequently used in the diagnosis and management of liver diseases. These enzymes are present in high concentrations in hepatocytes, while they leak into the circulation when hepatocyte membranes are damaged (Kew, 2000). As observed in our experiment, CCl_4 treatment caused a significant and drastic increase in serum ALT, AST, and LDH activities in rats. Pretreatment with AE-PA in rats at a dose of 500 mg/kg b.w. significantly reversed these trends, indicating that AE-PA possesses potent hepatoprotective activity *in vivo*.

The changes in serum enzyme activities reflect the extent of cell damage; this can be confirmed by histopathological examinations. Carbon tetrachloride treatment results in massive changes of fat, necrosis, lymphocyte infiltration, loss of cellular boundaries, and condensation of nuclei in liver sections (Liang *et al.*, 2011). We observed that AE-PA was able to reduce cell necrosis and minimize inflammation, which supported the biochemical results.

Oxidative stress is usually involved in liver diseases (Huang *et al.*, 2010). Therefore, in the present study, we evaluated the antioxidant activity of AE-PA. Hydroxyl radicals have the strongest chemical reactivity among various reactive oxygen species (ROS); they can damage a wide range of essential biomolecules, such as amino acids, proteins, and DNA (Halliwell and Gutteridge, 1990). The superoxide anion radical is also one of the most common free radicals generated *in vivo*, and is one of the precursors of singlet oxygen and hydroxyl radicals. Excessive production of superoxide anion radicals is regarded the onset of ROS accumulation in cells, resulting in harmful physiological consequences (Li *et al.*, 2010). Our results showed that AE-PA has good hydroxyl radical and superoxide anion radical scavenging activity. At 50 mg/mL AE-PA, the relative hydroxyl radical and superoxide anion radical scavenging activities were both over 60%.

The inhibitory effect of AE-PA on the increase of hexobarbital-induced sleeping time, and AST, ALT, and LDH levels demonstrated its hepatoprotective activity, which is also supported by the histopathological observations. Experiments on the hydroxyl radical and superoxide anion radical scavenging activities indicated a good antioxidant activity of AE-PA, which possibly contributed to its hepatoprotective activity. It has been reported that there are large quantities of phenolic acids, alkaloids, triterpenes, steroids, and glucose in *P. australis* (Gao *et al.*, 2009; SATCM, 1999). These bio-active components might play important roles in the hepatoprotective activity of AE-PA. Hence, more research is needed to identify the bio-active compounds in *P. australis* and to screen this potential drug for prevention and treatment of liver damage.

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