1 Protective effect of a polysaccharide from *Rhizoma Atractylodis Macrocephalae*
2 on acute liver injury in mice

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15 Abstract

16 A homogeneous polysaccharide was isolated and purified from *Rhizoma Atractylodis*
17 *Macrocephalae* (RAM) and named PRAM2. Its average molecular weight was $19.6 \times$
18 10^3 Da and it was composed of rhamnose, xylose, arabinose, glucose, mannose and
19 galactose in a ratio of 1: 1.3: 1.5: 1.8: 2.1: 3.2. In vitro experiments confirmed that
20 PRAM2 presented an obvious effect to scavenge 1,1-Diphenyl-2-picrylhydrazyl
21 radical 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) (DPPH), superoxide anion and hydroxyl
22 radical. In vivo experiments confirmed that PRAM2 could reduce the liver weight,

liver index, aspartate transaminase (AST) and alanine aminotransferase (ALT) activities in the serum; meanwhile, PRAM2 could significantly reduce nitric oxide synthase (NOS) activity, and nitric oxide (NO) and malonaldehyde (MDA) contents in the liver tissues, and increase superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. These results suggest that PRAM2 has a significant in vitro antioxidant activity and a protective effect on CCl₄-induced liver injury in mice; the protective effect may be related to its anti-oxidation, its inhibition of NOS activity and NO level and its reduction of the production of free radicals.

Key words: *Rhizoma Atractylodis Macrocephalae*; Polysaccharide; Liver injury

1. Introduction

The liver is the largest organ for biological metabolism in the body and also the main target organ attacked by exogenous substances and their metabolites [1]. Exogenous chemicals up-taken by the body through various routes, and toxic and harmful substances generated in the metabolism of body, eventually are transformed and stored in the liver, and these harmful toxic substances can cause acute and chronic liver injuries, causing liver necrosis, apoptosis, inflammation, fibrosis, carcinogenesis and other pathological changes [2]. Hepatic disease is one of the most common diseases to affect human health, but so far, the pathogenesis of acute and chronic liver injury is still not fully understood, and no confirmed effective drug used for the treatment of it has been discovered yet. It is considered that the present therapies of hepatic diseases can remove the pathogenic factors, protect the liver normal structure and function, correct a variety of the pathophysiological states and directly improve

the clinical symptoms [3]. Currently, the prevention and treatment of hepatic diseases have attracted the attention of the world, so that how to effectively prevent and persist in developing and discovering more effective and inexpensive hepatoprotective drug has become a consensus of many pharmaceutical workers [4, 5]. Based on the clinical and experimental research, the therapeutic effect of Chinese medicines on liver injury is recognized to be exerted by their protective effect on liver injury, including to recover the liver function, improve the liver pathology, modulate the immunity and be against hepatitis B virus, and it is believed that some herbal medicines can improve the liver function, alleviate the liver lesions, facilitate the recovery of liver cells, even inhibit the liver fibrosis [6]. Clinical advantages of Chinese medicines for the treatment of liver injury have been increasingly recognized and paid more attention by domestic and foreign experts [7].

Rhizoma Atractylodis Macrocephalae (RAM), a Chinese herbal medicine, is the dried root of a Compositae plant, and one of **important medicinal plant** in China and other Asian countries, which has long been widely used as a digestive, diuretic, and antihidrotic [8]. Modern pharmaceutical research has demonstrated that RAM and its main compounds could exert multifarious pharmaceutical effects in experimental models, including anti-hepatotoxicity, anti-inflammation, anti-diabetic activity, neuroprotective effect, immunoregulation and many other pharmacological activities [9-13]. Polysaccharides widely exist in roots, stems, leaves, flowers, fruits and seeds of plants, and are considered to have antioxidant, immunomodulating, anti-tumor, anti-allergic, anti-aging, anti-virus and other biological activities, but with less and

weak toxic side effects, and high safety, so that they have been widely used in clinic [14-16]. The research on activities of RAM polysaccharide primarily focuses on its improvement of the immune system and myocardial protection activities, but there is less related report now to the best of our knowledge [17]. Given that the biological activity of plant polysaccharides is with a diversity, especially a large number of studies have reported that they can protect liver injury [18-20], a homogeneous polysaccharide named PRAM2 was isolated and purified from the roots of RAM, and its antioxidant activity in vitro and protective effect on CCl₄-induced liver injury model in mice were investigated in this study.

2. Materials and methods

2.1. Materials and chemicals

The roots of RAM were purchased from the local drugstore in China. Trifluoroacetic acid (TFA), T-series dextrans (T-2000, T-70, T-40, T-20, and T-10), the standard monosaccharides (rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose) and DPPH were purchased from Sigma Chemical Co. (MO, USA). The test kits of super oxygen anion radical, hydroxyl radical, AST, ALT, SOD, GSH-Px and MDA were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). NOS and NO levels were determined using an ELISA kit of R&D system Inc. (MN, USA).

2.2. Extraction, isolation and purification of PRAM2

To remove the fat-soluble impurities, the crude dried roots of RAM were processed 3 times with petroleum ether reflux extraction (60-90°C) and each time lasted for 6 h,

and the petroleum ether was recycled by rotary evaporation. The dried drug residue was soaked with 80% ethanol overnight, then it was processed 2 times with reflux method and 2 h for each time to remove the alcohol impurities, and the ethanol was recycled by rotary evaporation; distilled water was used to extract it 3 times and 2 h for each time; the mixed filtrate concentrated by rotary evaporation was dissolved in 4 volumes of absolute ethanol and it was kept at 4°C overnight; the solution was centrifuged and the collected precipitate was dried with a vacuum dryer. The dried precipitate was dissolved in distilled water and then Sevag reagent equal to 1/4 volume of the aqueous solution was added to it; the solution was shaken vigorously for 20 min in a separatory funnel and allowed to stand for stratified, which was repeated 3-5 times. After the deproteinization by Sevag method, it was dialyzed with water for 48 h, and then the dialysate was mixed with 4 volumes of absolute ethanol and placed at 4°C for 24 h. The mixed solution was concentrated for the collection of precipitate. The precipitate was washed successively with ethanol and acetone, and finally dried in vacuum to obtain a crude polysaccharide (named CPRAM).

CPRAM was dissolved in distilled water and loaded on an anionexchange column (3 cm × 50 cm) of DEAE-Sepharose Fast Flow ion exchange resin, and eluted stepwise as three fractions (CPRAM1, CPRAM2 and CPRAM3) with 0.1, 0.3, 0.5 and 0.7 M NaCl in Tris-HCl buffer. The major fraction CPRAM2 was further purified by Sephadex G-10 column with distilled water as the eluent. A purified polysaccharide (named PRAM2) was obtained.

2.3. Characterization of PRAM2

111 Using D-glucose as the reference substance, the total sugar content was determined
112 with phenol-sulfuric acid method [21]; using galacturonic acid as the reference, the
113 uronic acid content was measured with meta-hydroxydiphenyl method [22]; the
114 protein content was detected with Coomassie brilliant blue method [23].

115 *2.4. Monosaccharide composition analysis*

116 PRAM2 was hydrolyzed and acetylated according to Lehrfeld [24]. Simply, the
117 samples were hydrolyzed with TFA and then hydrolyzed product was reduced with
118 KBH_4 , followed by neutralization with acetic acid. After adding myo-inositol and
119 Na_2CO_3 , the residue was concentrated. The reduced products were added with
120 pyridine-propylamine and acetylated with pyridine-acetic anhydride. The acetylated
121 products were analyzed by GC, and identified and estimated with myoinositol as the
122 internal standard.

123 GC was performed on an Agilent 6890 instrument (Agilent Technologies, USA)
124 equipped with HP-5 capillary column ($30\text{ m} \times 0.32\text{ mm} \times 0.2\text{ }\mu\text{m}$), flame-ionization
125 detector (FID) and temperatures programmed from 120 to 250°C at a rate of $8^\circ\text{C}/\text{min}$.

126 The monosaccharide standards (rhamnose, fucose, arabinose, xylose, mannose,
127 galactose and glucose) were measured following the same procedure with myoinositol
128 as the internal standard.

129 *2.5. Molecular weight (MW) determination*

130 Molecular weight of PRAM2 was determined by high-performance size-exclusion
131 chromatography (HPSEC). The samples of polysaccharide fractions were dissolved in
132 distilled water, applied to a Shimadzu HPLC system equipped with a TSK-GEL

133 G3000 PWXL column, eluted with 0.1 mol/L Na₂SO₄ solution and detected by a
134 RID-10A refractive index detector. Dextran standards with different molecular
135 weights (T-2000, T-70, T-40, T-20, and T-10) were used to calibrate the column and
136 establish a standard curve.

137 2.6. Observation on the effect of PRAM2 on DPPH radical scavenging rate

138 1 mL of different concentrations of sample was put in a test tube, followed by adding
139 3 mL of methanol solution containing 0.004% DPPH to it, which was fully mixed and
140 kept in dark for 20 min for the absorbance measurement of it at 517 nm wavelength.
141 Instead of the sample solution, distilled water was taken as the negative control. The
142 scavenging rate of DPPH radical was calculated according to the following formula.

143
$$\text{Scavenging rate (\%)} = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100\%$$

144 A_{control} is the absorbance of control without the PRAM2 sample and A_{sample} is the
145 absorbance in the presence of the PRAM2 sample.

146 2.7. Observation on the effect of PRAM2 on superoxide anion radical scavenging rate

147 1 mL of the sample solution at different concentrations was put in a test tube, then 1
148 mL 0.078 mol/L NBT and 1 mL 0.468 mol/L NADH were added to them respectively,
149 and finally 0.4 mL of 0.06 mol/L PMS solution was added to them, which was shaken
150 fully at room temperature and kept for 5 min for the measurement of the absorbance
151 at 560 nm wavelength. Distilled water was taken as the negative control instead of the
152 sample solution and the superoxide anion radical scavenging rate was calculated
153 according to the formula described in section “2.6”.

154 2.8. Observation on the effect of PRAM2 on hydroxyl radical scavenging rate

1.5 mL reaction solution (pH 7.4 phosphate buffer containing 0.1 mmol/L EDTA, 0.1 mmol/L FeCl_3 and 2.8 mmol/L DR) and 0.35 mL 20 mmol/L H_2O_2 were successively added to 1 mL of the sample solution at different concentrations, which was incubated in water at 37°C for 40 min, and then the reaction was terminated immediately. The absorbance was measured at 532 nm wavelength, in which distilled water took place of the sample solution to taken as the negative control and the hydroxyl radical scavenging rate was calculated with reference to the formula in section “2.6”.

2.9. Animals

Male ICR mice with 6-8 weeks old were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China) and acclimatized for 1 week before the experiments under a controlled light/dark cycle. The animals were kept in polypropylene cages in an air-conditioned room at 22-25°C and maintained on a standard laboratory feed and water ad libitum.

2.10. Grouping, administration and modeling

50 ICR mice were randomly divided into 5 groups, namely, a normal control group, model group, high-dose PRAM2 group (H-PRAM2), medium-dose PRAM2 group (M-PRAM2) and low-dose PRAM2 group (L-PRAM2). The mice were fed with the regular diet and allowed to access to water freely. Mice in H-PRAM2, M-PRAM2 and L-PRAM2 groups were intragastrically administered 200, 100 and 50 mg/kg PRAM2 one time daily for consecutive 15 d, respectively, and those in the normal control and model groups were given the same volume of distilled water in the same way. At 1 h after the last administration, mice in the model group and different doses of

PRAM2-treated groups were intraperitoneally injected 2.0 mL/kg body weight of 10% CCl₄, those in the normal control mice were injected the same volume of olive oil, and all the mice were fasted, but allowed freely to access to water.

2.11. Determination of biochemical indicators

On the 6th hour after the modeling, the blood samples were taken with removalling eyeball, and the amount of sample from each mouse was about 0.8-1.0 mL. The blood was centrifuged to separate the serum. The separated serum samples were put into eppendorf tubes, separately, which were kept at -70°C for cryopreservation. According to the procedure of kit instructions, serum ALT and AST activities were measured. Mice were sacrificed by cervical broking method to immediately remove their livers. The liver samples were washed with cold saline to remove the blood and dried with filter paper. Then, the liver weight was weighed and the liver index was calculated according to the following formula.

$$\text{Liver index} = \text{liver weight (g)} / \text{body weight (g)} \times 100\%。$$

The liver tissue in an appropriate amount of ice saline was prepared into a homogenate containing 10% liver tissue. The homogenate was centrifuged at 3500 rpm and 4°C for 15 min to obtain the supernatant. According to the kit instructions, NOS, SOD and GSH-Px activities, and NO and MDA levels in the liver homogenate were measured.

2.12. Histological examinations

The liver tissue at the same position of the right lobe of mice was taken and saline at 4°C was used to wash away the residual blood on it and then dried with a filter paper.

It was fixed with 10% neutral formalin, embedded with paraffin, sectioned and stained with hematoxylin-eosin. Histoathological changes in the liver tissue sections were observed under a light microscope.

2.13. Statistical analysis

Data were expressed as mean $\pm$ standard deviation (S.D.). Student's *t*-test and analysis of variance (ANOVA) were used to determine the statistical significance (Package for Social Sciences version 15.0, SPSS Inc., Chicago, IL, USA). A *P* value less than 0.05 or 0.01 was considered significant.

3. Results and Discussion

3.1. Isolation and purification of PRAM2

The crude polysaccharide was obtained from the roots of RAM by distilled water extraction. The whole extract was precipitated in 80% (v/v) ethanol. The precipitate was then centrifuged, and dried in vacuum. The yield of crude polysaccharide to the dry weight of roots of RAM was 2.86%.

The crude polysaccharide was isolated by DEAE-Sepharose Fast Flow anionexchange chromatography and the chromatogram showed three peaks. The major large peak 2 eluted by 0.3 M NaCl was further purified by Sephadex G-10 column, successively. Only a sharp peak was detected in the Sephadex G-10 column chromatography. Then the sharp peak was collected and named as PRAM2.

3.2. Characterization, monosaccharide composition, molecular weight in PRAM2

The total sugar content and uronic acid content of PRAM2 were 81.9% and 18.1%. Coomassie brilliant blue method showed that PRAM2 was free of protein. The

221 HPSEC analysis of PRAM2 exhibited a single and symmetrical peak, basically
222 declaring the homogeneity of the polysaccharide. Its molecular weight was
223 determined as 19.6×10^3 Da. The GC analysis showed that PRAM2 contained
224 rhamnose, xylose, arabinose, glucose, mannose and galactose. The molar ratio of the
225 monosaccharides was calculated from the chromatogram area ratios and was found to
226 be 1: 1.3: 1.5: 1.8: 2.1: 3.2 (rhamnose: xylose: arabinose: glucose: mannose:
227 galactose).

228 *3.3 In vitro antioxidant activity of PRAM2*

229 PRAM2 presented a significant activity to scavenge DPPH, superoxide anion and
230 hydroxyl radical, and the activity was obviously dose-dependent, especially when the
231 mass concentration reached 1 mg/mL, the effect of PRAM2 on DPPH radical,
232 superoxide anion radical and hydroxyl radical scavenging rate reached 75.4%, 50.1%
233 and 54.4%, respectively. The results were shown in Table 1.

234 Preferred position of Table 1

235

236 *3.4 Effects of PRAM2 on the body weight, liver weight and liver index of mice with* 237 *CCL₄-induced liver injury*

238 Compared with the normal control group, there was no significant difference in body
239 weight in the other groups, while the liver weight and liver index of mice in the model
240 group were significantly increased ($P < 0.01$), indicating that the modeling was
241 successful. As shown in Table 2, compared with those in the model group, the liver
242 weight and liver index of mice in H-PRAM2 and M-PRAM2 groups were

significantly lower ($P < 0.01$ or $P < 0.05$), but those in L-PRAM2 group did not significantly change ($P > 0.05$).

Preferred position of Table 2

3.5 Effects of PRAM2 on serum AST and ALT activities of mice with CCl₄-induced liver injury

Serum AST and ALT activities of mice in the model group were significantly greater than those in the normal control group, suggesting that the modeling was successful; compared with those in the model group, serum AST and ALT activities of H-PRAM2, M-PRAM2 and L-PRAM2 groups were significantly reduced ($P < 0.01$ or $P < 0.05$), which presented a dose-dependent manner. The results are shown in Figure 1.

Preferred position of Figure 1

3.6 Effects of PRAM2 on the NOS activity and NO content in liver tissues of mice with CCl₄-induced liver injury

Compared with those in the model group, the NOS activity and NO content in the liver tissue of mice in the normal control group were significantly reduced; those in high-, medium- and low-dose PRAM2 groups were also significantly reduced ($P < 0.01$ or $P < 0.05$), which were significantly dose-dependent. (Table 3)

Preferred position of Table 3

265 3.7 Effects of PRAM2 on SOD and GSH-Px activities and MDA content in liver
266 tissues of mice with CCl₄-induced liver injury

267 SOD and GSH-Px activities in the liver tissue of mice in the model group were
268 significantly lower than those in the normal group, and compared with those in the
269 model group; SOD and GSH-Px activities in the liver tissue of mice in H-PRAM2,
270 M-PRAM2 and L-PRAM2 groups were significantly increased ($P < 0.01$); MDA
271 content in the liver tissue of mice in the model were significantly higher than those in
272 the normal group, and compared with those in the model group, MDA content in the
273 liver tissue of mice in H-PRAM2, M-PRAM2 and L-PRAM2 groups were
274 significantly lower ($P < 0.01$). The results are shown in Figure 2.

275 Preferred position of Figure 2

276
277 3.8 Effects of PRAM2 on histopathological changes in liver tissues of mice with
278 CCl₄-induced liver injury

279 The structure of liver lobule was intact, the hepatic cord arranged neatly and regularly,
280 the structure and the morphology of liver cells were normal, the cytoplasm was
281 abundant, the nucleus was large and round, the distribution of nucleoplasm was
282 uniform and the nucleolus was clear in mice in the normal group. In mice in the
283 model group, the damage of liver cells was obvious, showing a significant swelling of
284 liver cells, unequal size and different shrinkage of the nucleus, loosen cytoplasm,
285 disorder of hepatic cord arrangement, punctate and piecemeal necrosis, extensive
286 infiltration of inflammatory cells. Compared with that in the model group, the damage

of liver cells was significantly alleviated in PRAM2-treated groups, in which the arrangement of liver cells was neater, no necrotic tissue was found, a mild edema of some hepatocytes could be only seen and less inflammatory infiltration area could be found; the damage of liver cells in H-PRAM2 group was mildest, in which the damage was least and no inflammatory infiltration could be seen. These results suggest that PRAM2 has a significantly protective effect on CCl₄-induced liver cell injury in mice.

Preferred position of Figure 3

4. Discussion

DPPH radical is a very stable nitrogen-centered radical and its lone pair of electrons shows a strong absorption near 517 nm wavelength. When a radical scavenger exists, the lone pair of electrons are paired and the absorption disappeared or decreased; by measuring the weakened extent of absorption, the antioxidant activity of antioxidants can be evaluated [25]. Superoxide anion radical, as the precursor of a variety of reactive oxygen species, can cause a damage to organisms; superoxide anion radical can be produced in the body and its oxidation is weaker, but it can be broken down into a singlet oxygen radical or hydroxyl radical with high oxidation, and these singlet oxygen and hydroxyl radical can break a single-stranded DNA [26]. Hydroxyl radical is the most active oxygen species in the body and can cause a variety of diseases, suggesting that the detection of scavenging antioxidant activity of hydroxyl radical is also one of important indicators for antioxidant activity [27]. The radical scavenging

309 rate of PRAM2 on DPPH, superoxide anion and hydroxyl radical were investigated in
310 this study and the results showed that PRAM2 should have a good in vitro antioxidant
311 activity.

312 CCl₄ can directly damage liver cells to cause a series of specific changes in them,
313 in which CCl₄ can be metabolized into CCl₃ and Cl radicals in the liver, and these two
314 kinds of radicals can damage lipid and protein macromolecules of the cell membrane
315 to result in metabolic disorders and lipid peroxidation, thereby increasing the
316 permeability of cell membrane and leading to the outflow of AST and ALT from the
317 cells under normal circumstances [28, 29]. Under normal circumstances, AST and
318 ALT are intracellular enzymes and only little of them exists in serum, but AST and
319 ALT can be released to the outside of cells when the cells are damaged, leading to an
320 increased permeability of the cell membrane, thus resulting in the increase in serum
321 AST and ALT contents [30]. This study demonstrated that PRAM2 could reduce the
322 liver weight and liver index, AST and ALT activities in the serum, and indicating an
323 apparent protection of it on the acute liver injury model induced by intraperitoneally
324 injecting 10% CCl₄; moreover, the observation on histopathological changes of the
325 liver could further confirm the protective effect of PRAM2 on the liver.

326 Free radical and lipid peroxidation are important factors to cause liver damage.
327 Free radical is an intermediate product in metabolic processes of the body and lipid
328 peroxidation is a series of reactions triggered by free radicals. NOS is a key enzyme in
329 the synthesis of NO in the body and can catalyze L-arginine to generate NO [31]. NO
330 can show a double effect on the liver, that is, physiological concentrations of NO can

present a protective effect on the liver, but high concentrations of it can react with reactive oxygen species of the body to generate peroxynitrite radicals with a strong cytotoxicity, which can inhibit the synthesis of liver cellular proteins and damage the structure of DNA to cause the disorder of energy metabolism in liver cells, leading to the apoptosis and necrosis of liver cells [32]. Our study proved that the pre-administration of PRAM2 can lower NOS activity and NO content in the liver tissues of mice so as to weaken acute liver injury.

SOD and GSH-Px, important antioxidant enzymes in the body, can effectively remove free radicals produced by the body to inhibit free radical-initiated lipid peroxidation [33]. When liver cells are attacked by free radicals, SOD and GSH-Px can be reduced because of the excessive consumption, so that it can be understood that the higher the activity of SOD and GSH-Px in the body is, the faster the velocity of radical scavenging is [34, 35]. MDA, the final product generated in the metabolism of lipid peroxides when lipids are attacked by free radicals, can further damage the cells, which can not only reflect the degree of sensitivity of lipid peroxidation but also indirectly reflect the degree of cell injury [36]. It is illustrated in this study that the pre-administration of PRAM2 can increase SOD and GSH-Px activities, and reduce MDA content in the liver tissues, suggesting that the anti- acute liver injury function of PRAM2 is may be related to its function of anti-oxidative stress.

The maintenance in calcium homeostasis plays a vital role in the initiation and implementation process of liver cell injury and death [37]. In the liver injury induced by ischemia-reperfusion, the intracellular free calcium concentration ($[Ca^{2+}]_i$) is rapidly elevated when the reperfusion of anoxic hepatocytes induce the reoxygenation,

and the persistent elevated $[Ca^{2+}]_i$ can cause the necrosis and apoptosis of liver cells [38, 39]. In the viral liver injury, HBV DNA (HBx gene) can bind to the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) to encode a chimeric protein, which can break the normal SERCA protein, so that SERCA protein can lose its ability to pump the cytosolic Ca^{2+} into the endoplasmic reticulum (ER) to cause an increase in cytosolic Ca^{2+} content and a transfer of it into the mitochondria, thereby promoting the apoptosis of liver cells [40]. In the CCl_4 -induced chemical liver injury, CCl_4 can be metabolized by the cytochrome P-450 into CCl_3 free radicals with more activity to result in a destruction of calcium homeostasis, ultimately causing the death of liver cells [41]. It can be concluded that various types of liver damage are associated with the destruction of calcium homeostasis. The related studies have showed that the protective effect of liver injury is associated with the regulation of calcium homeostasis [42-44]. Due to this reason, we will focus on whether PRAM2 can protect the liver from being damaged by affecting the calcium homeostasis in the future study.

5. Conclusion

In this study, a bioactive polysaccharide PRAM2 from RAM was successfully isolated and characterized. Its average molecular weight was 19.6×10^3 Da and it was composed of rhamnose, xylose, arabinose, glucose, mannose and galactose in a ratio of 1: 1.3: 1.5: 1.8: 2.1: 3.2. The results indicate that PRAM2 has a certain protective effect on the CCl_4 -induced liver injury in mice; the protective effect may be related to its anti-oxidation, its inhibition of NOS activity and NO level, and its reduction in the production of free radicals.

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Table 1 In vitro antioxidant activity of PRAM2

Mass concentration (mg/mL)	Free radical scavenging rate (%)		
	DPPH	Superoxide anion	Hydroxyl
0.2	17.5	21.1	17.7
0.4	21.3	34.4	31.2
0.6	44.6	41.2	40.9
0.8	61.7	47.8	48.5
1.0	75.4	50.1	54.4

Table 2 Effects of PRAM2 on the body weight, liver weight and liver index of mice with CCl₄-induced liver injury.

Group	Dose (mg/kg)	Body weight (g)	Liver weight (g)	Liver index (%)
Normal	—	28.34 ± 3.24	1.35 ± 0.14 ^b	4.76 ± 0.45 ^b
Model	—	28.61 ± 3.05	1.59 ± 0.19	5.56 ± 0.52
H-PRAM2	200	28.52 ± 3.43	1.42 ± 0.15 ^b	4.98 ± 0.49 ^b
M-PRAM2	100	27.98 ± 3.61	1.51 ± 0.21 ^a	5.40 ± 0.52 ^a
L-PRAM2	50	28.09 ± 3.32	1.55 ± 0.16	5.51 ± 0.44

Each value is presented as mean ± SD (n = 10). ^a: $P < 0.05$, significantly different from those in the model group, ^b: $P < 0.01$, significantly different from those in the model group.

Table 3 Effects of PRAM2 on NOS activity and NO content in liver tissues of mice
with CCl₄-induced liver injury.

Group	Dose (mg/kg)	NOS (U/mgprot)	NO (μg/gprot)
Normal	—	2.61 ± 0.52 ^b	4.06 ± 0.45 ^b
Model	—	3.45 ± 0.63	7.69 ± 0.82
H-PRAM2	200	2.73 ± 0.48 ^b	5.12 ± 0.68 ^b
M-PRAM2	100	2.94 ± 0.44 ^a	5.66 ± 0.76 ^b
L-PRAM2	50	3.15 ± 0.39 ^a	6.24 ± 0.93 ^a

Each value is presented as mean ± SD (n = 10). ^a: $P < 0.05$, significantly different from those in the model group; ^b: $P < 0.01$, significantly different from those in the model group.

Figure 1 Effects of PRAM2 on AST and ALT activities in serum of mice with CCl₄-induced liver injury.

Figure 2 Effects of PRAM2 on SOD and GSH-Px activities, and MDA content in liver tissues of mice with CCl₄-induced liver injury.

Figure 3 Effects of PRAM2 on histopathological changes in liver tissues of mice with CCl₄-induced liver injury.

Figure 1 Effects of PRAM2 on AST and ALT activities in serum of mice with CCl₄-induced liver injury. Each value is presented as mean $\pm$ SD (n = 10); ^a: $P < 0.05$, compared with those in the model group; ^b: $P < 0.01$, compared with those in the model group.

Figure 2 Effects of PRAM2 on SOD and GSH-Px activities, and MDA content in liver tissues of mice with CCl₄-induced liver injury. Each value is presented as mean $\pm$ SD (n = 10); ^a: $P < 0.05$, significantly different from those in the model group; ^b: $P < 0.01$, significantly different from those in the model group.

Figure 3 Effects of PRAM2 on histopathological changes in liver tissues of mice with CCl₄-induced liver injury (×100).