

HPLC Analysis, Optimization of Extraction and Purification Conditions, Biological Evaluation of Total Protoflavones from *Macrothelypteris viridifrons*

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ABSTRACT:

The present study aims to evaluate phytochemical and pharmacological potential of total protoflavones from *Macrothelypteris viridifrons*. In the phytochemical study, an HPLC analysis method was established, and the optimal extraction and purification conditions were analyzed. The extractive condition was optimized as follows: the backflow extraction with 20 folds of 70% ethanol at 80°C for 1 h twice. Moreover, by combining the alkali-extraction and acid-precipitation method with the macroporous resin purification technology, the final purity rate of total protoflavones was no less than 54.85%. In the pharmacological study, the total protoflavones from *M. viridifrons* showed a significant tumor-inhibitory effect in the H22 hepatoma cells transplantation model with a higher inhibitory rate of 55.76% in high dosage (100mg/kg) treatment group compared with the positive control group (20 mg/kg cyclophosphamide). Taken of all, these results support that protoflavones are the material basis of *M. viridifrons* as an anticancer folk medicine.

Keywords: *Macrothelypteris viridifrons*; HPLC; Protoflavone; Protoapigenone; Anti-tumor;

Figure S1. Chemical structures of protoapigenone.

Figure S2. The HPLC-UV chromatograms for (A) the standard solution; (B) the extract of *M. viridifrons* (P: Protoapigenone).

Figure S3. The purified flow chart of total protoflavones from *M. viridifrons*.

Figure S4. The HPLC-UV chromatograms for (C) the fraction by the alkali-extraction and acid-precipitation method; (D) the fraction by the macroporous resin purification technology (P: Protoapigenone).

Table S1. Analytical results of the HPLC Method Validation.

Table S2. The results of orthogonal test for the optimization of extraction conditions.

Table S3. The process validation for purification of total protoflavones from *M. viridifrons*.

Table S4. Tumor weight, tumor inhibitory rate, body weight (BW) profile, complete blood count and biochemical profile after treatment with total protoflavones from *M. viridifrons* for 10 days.

2. Experimental

2.1 Chemicals and Animals

Methanol and ethanol were purchased from Tianjin Kemiou Instrument Factory (Tianjin, China). Different types of macroporous resin were purchased from Changzhou Baoen Chemical Factory (Hebei, China).

The H22 tumor cells and the male Kunming mice (18-22 g) were provided by the College of Pharmacy, Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). All the procedures were approved by Animal Ethical Council of Huazhong University of Science and Technology. All animals fed with food and water *ad libitum* in controlled conditions (12 h light/dark cycle, 24 ± 1 °C and humidity of $55 \pm 5\%$).

2.2 Plant Material

The rhizomes of *M. viridifrons* were collected from Lushan in China and identified by Prof. Chang-Gong Zhang, College of Pharmacy, Huazhong University of Science and Technology. A voucher specimen (MV0906) was deposited in the College of Pharmacy, Tongji Medical Center, Huazhong University of Science and Technology. The reference substance confirmed 5, 7-dihydroxy-2-(1-hydroxy-4-oxocyclohexa-2, 5-dienyl)-chromen-4-one (protoapigenone), which was isolated from *M. viridifrons* in our experiment by repeated column chromatography and identified by spectrometric parameters (UV, MS and NMR) (Wei et al., 2011a). The purity of protoapigenone was found to be greater than 97% based on the percentage of peak area by HPLC analysis.

2.3 Phytochemical Studies by the HPLC Analysis

2.3.1 Instrumentation and Chromatographic Conditions

The HPLC analysis system (Hitachi, Japan) was equipped with a Model L-2130

pump, an on-line solvent vacuum degasser, an auto sampler with 20 μ L injection loop, an L-2400 UV detector, an Agilent TC-C₁₈ column (250 mm \times 4.6 mm, 5 μ m) and the T2000P software. Chromatographic separations were carried out on the binary gradient elution system, consisting of methanol (A) and water (B) with the following gradient program: 0 -10 min (15- 30% A), 10- 25min (30% A), 25- 35 min (30- 40% A), 35-45 min (40- 45% A), 45- 60 min (45- 65% A). The flow rate was set at 1.0 ml/min, the column temperature was 25 $^{\circ}$ C, the detection wavelength was 262 nm and the injection volume was 20 μ l.

2.3.2 Standards and Samples Preparation

Protoapigenone (5.2 mg) was dissolved in a 10 mL volumetric flask with methanol as the mixed standard stock solution, and then the stepwise dilution was performed to prepare the working standards for the concentration of the calibration range.

The rhizomes of *M. viridifrons* were dried at room temperature and crashed into powder (20 meshes). The resulting powder was accurately weighed (about 1g) and then was extracted with 50 ml methanol in an ultrasonic water bath for 40 min at room temperature. The extract was filtered through a 0.45 μ m polytetrafluoroethylene membrane filter (Tianjin Jinteng Instrument Factory, Tianjin, China) for qualitative analysis.

2.4 Preparation of Total Protoflavones

2.4.1 Optimization of Extraction Conditions

For obtaining optimal extraction efficiency of *M. viridifrons*, the orthogonal test was designed and four relative extractive conditions were evaluated, which included the following factors and corresponding levels: (A) concentration of ethanol (60%, 70% and 80%), (B) extraction temperature (60, 70 and 80 $^{\circ}$ C), (C) extraction time (0.5, 1 and 1.5 h) and (D) solid-liquid ratio (1:10, 1:20 and 1:30).

2.4.2 Optimization of Purification Conditions

Considering the special chemical structure characteristics of protoflavones, the alkali-extraction and acid-precipitation method was firstly applied to purify. The following factors and corresponding levels were taken into account: (A) the PH value of the extraction with strong ammonia (5, 6, 7 and 8), (B) the PH value of the precipitation with hydrochloric acid (1, 2, 3 and 4). And then the filtrate fraction was purified with macroporous resin technology and involved the following variables: (A) the optimal type of macroporous resin; (B) the optimal capability of adsorption, including static adsorption kinetics, dynamic adsorption kinetics, the PH value of solution (3, 4, 5, 6 and 7), the concentration of solution (2.0mg/mL, 4.0mg/mL and 6.0mg/mL) and the flow velocity of solution (1.0BV/h, 2.0BV/h and 3.0BV/h); (C) the optimal capability of desorption, including dynamic desorption kinetics, the concentration of eluent (10%, 30%, 50%, 70% and 90%) and the flow velocity of eluent (1.0BV/h, 2.0BV/h and 3.0BV/h). By comparing the sum numbers and areas of target peaks in each chromatogram profile, the optimal purified conditions were confirmed.

2.5 *In Vivo* Pharmacological Studies

The H22 hepatoma cells maintained in the ascites of mice were injected into the right flank of each mouse for establishing a xenograft model (Wu et al., 2010). All mice were randomly divided into 5 groups after 24 h. The total protoflavones from *M. viridifrons* were diluted in 0.9% normal saline. The dosages administered were 25 mg/kg, 50 mg/kg, and 100 mg/kg. In addition, equivalent 0.9% normal saline and 20 mg/kg of cyclophosphamide (CTX) were used as the vehicle and positive controls, respectively. All groups were administrated every day and lasted for 10 days. At the end of treatments, the mice were sacrificed and their body, tumor, liver, kidney, spleen and thymus weights were measured. Tumor inhibitory rate was calculated by the following equations: inhibition rate (%) = $(A-B)/A \times 100\%$ (A and B were the

average tumor weights of the treated and vehicle control mice, respectively). Meanwhile, the blood biochemical indexes, including the red blood cell (RBC), white blood cell (WBC), platelet (PLT), haemoglobin (HGB), blood urea nitrogen (BUN), serum creatinine (Cr), glutamic-oxaloacetic transaminase (AST) and glutamic-pyruvic transaminase (ALT), were also determined in the present study.

2.6 Statistical analysis

All values are presented as the means \pm standard deviation (SD). Data were analyzed by using SPSS 20.0 software (SPSS, USA). Statistical significance was assessed by one-way ANOVA or Student's t-test. Differences with $p < 0.05$ were considered to be statistically significant.

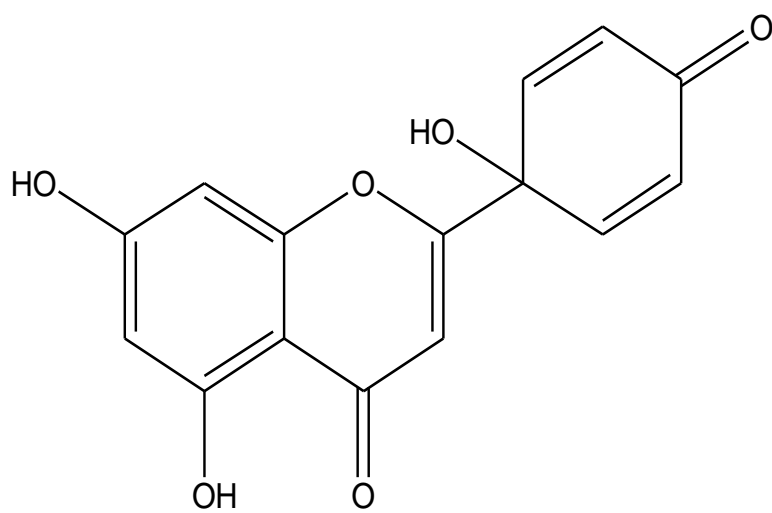


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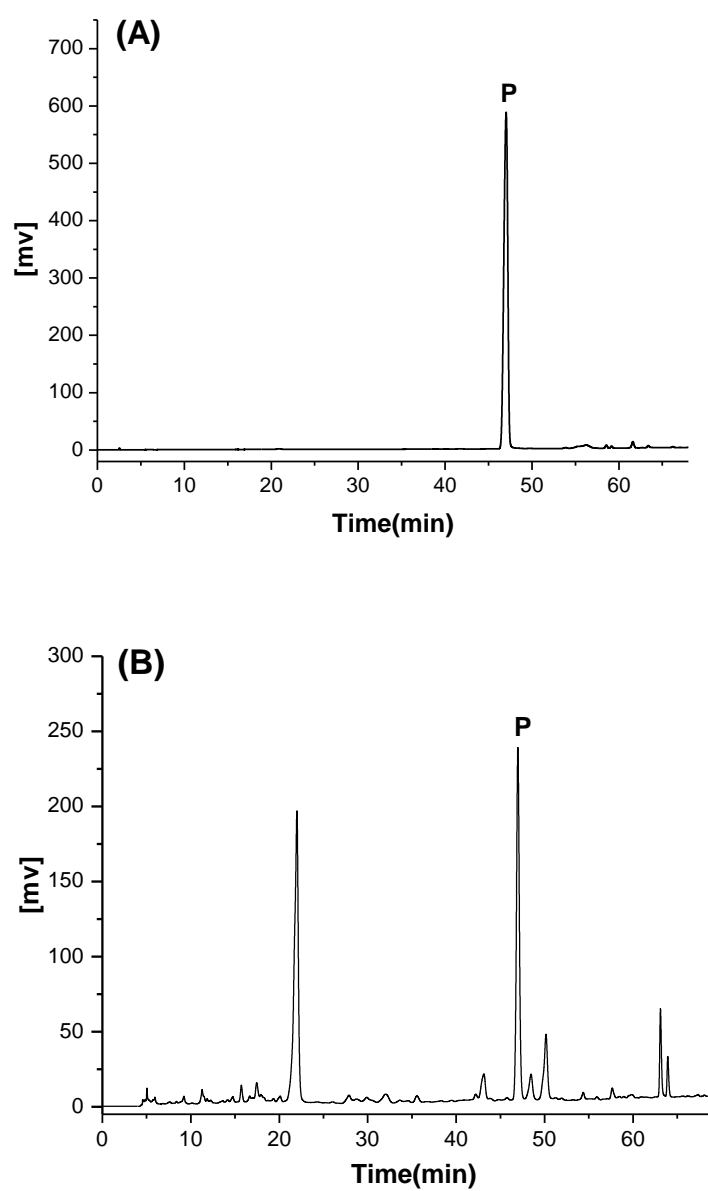


Figure S2. The HPLC-UV chromatograms for (A) the standard solution; (B) the extract of *M. viridifrons* (P: Protoapigenone).

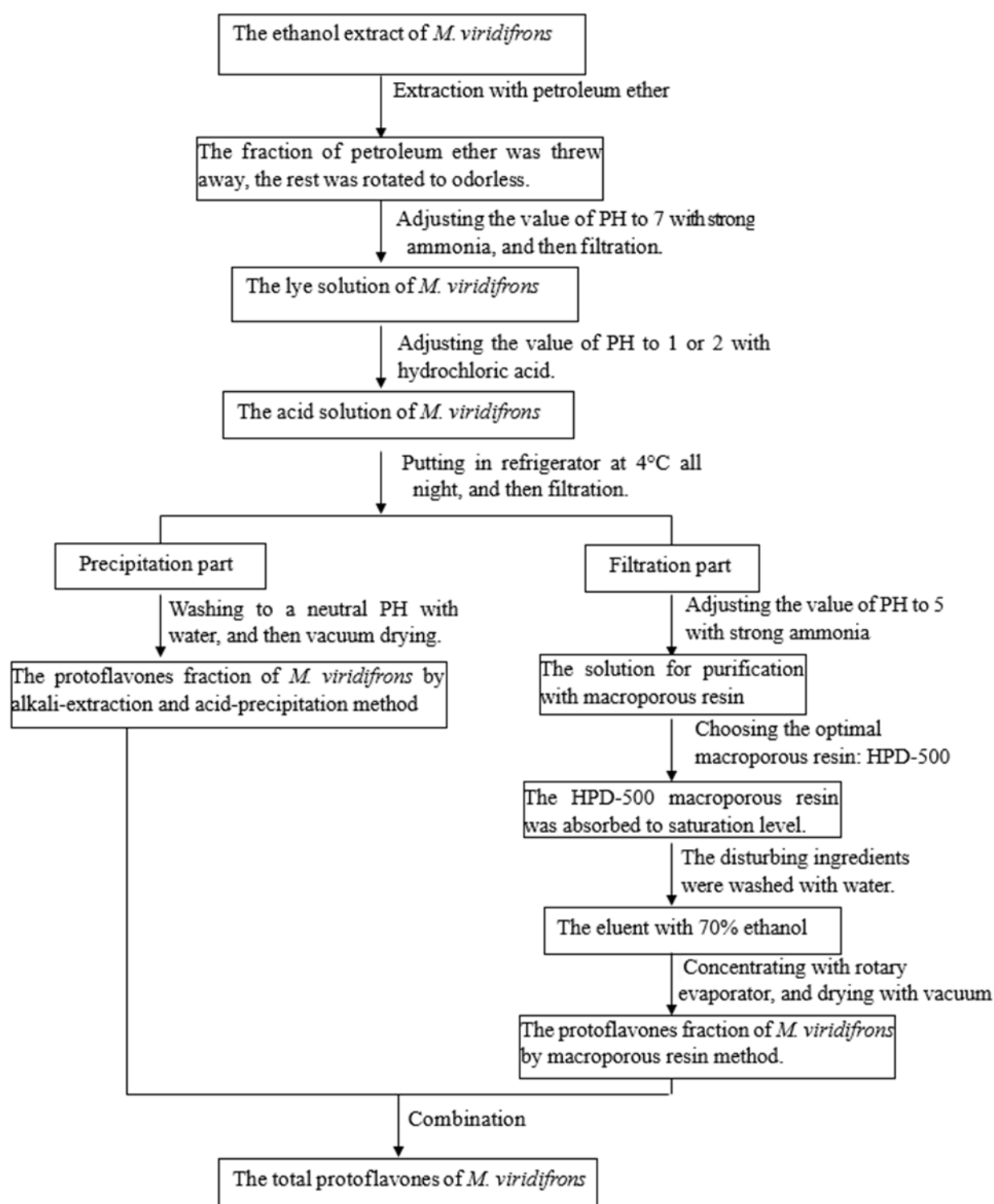


Figure S3. The purified flow chart of total protoflavones from *M. viridifrons*.

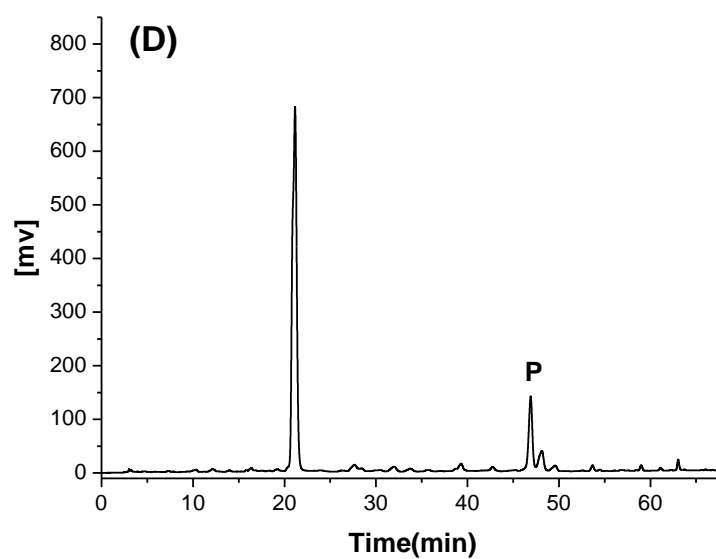
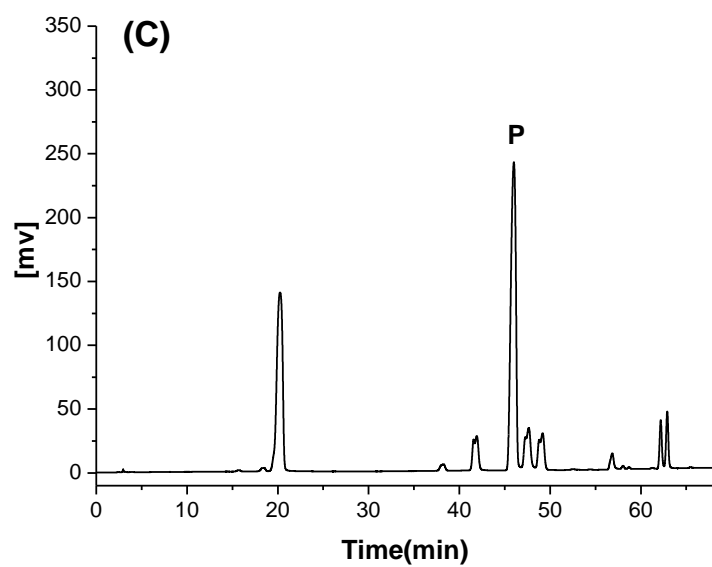


Figure S4. The HPLC-UV chromatograms for (C) the fraction by the alkali-extraction and acid-precipitation method; (D) the fraction by the macroporous resin purification technology (P: Protoapigenone).

Table S1. Analytical results of the HPLC Method Validation

Linear correlation	$y = 30277x - 202335, r = 0.9998$		
LOD ($\mu\text{g/mL}$)	1.625		
LOQ($\mu\text{g/mL}$)	3.954		
Precision assay	protoapigenone		
Concentration ($\mu\text{g/mL}$)	50	150	250
Intra-day (n=3)			
Accuracy (%)	99.56	102.12	98.67
RSD (%)	0.98	1.15	1.35
Inter-day (n=3)			
Accuracy (%)	100.98	103.22	98.41
RSD (%)	1.29	1.88	2.12
Inter-day (repeatability)			
Accuracy (%)	98.56	99.58	100.05
RSD (%)	1.28	1.02	0.67
Recovery assay	protoapigenone		
Added Concentration ($\mu\text{g/mL}$)	50	150	250
Recovery (%) \pm SD	97.6 \pm 0.85	96.9 \pm 0.98	98.0 \pm 1.07
RSD (%)	1.82	2.06	1.74

Table S2. The results of orthogonal test for the optimization of extraction conditions

Number	(A)%	(B)°C	(C)min	(D)g/ml	content(mg/g)
1	60	60	30	1: 10	6.98
2	60	70	60	1: 20	10.75
3	60	80	90	1: 30	10.20
4	70	60	60	1: 30	10.35
5	70	70	90	1: 10	5.96
6	70	80	30	1: 20	12.47
7	80	60	90	1: 20	8.25
8	80	70	30	1: 30	8.19
9	80	80	60	1: 10	8.75
k1	3.131	3.053	3.121	2.923	
k2	3.159	3.030	3.195	3.249	
k3	3.040	3.247	3.014	3.158	
R	0.119	0.217	0.181	0.326	

Table S3. The process validation for purification of total protoflavones from *M. viridifrons*.

Sample	1	2	3	Average
Content of flavonoid in the ethanol extract of <i>M. viridifrons</i> (g)	7.05	7.02	6.89	
Weigh of precipitation (g)	7.64	7.42	7.72	
Content of flavonoid in the precipitation (g)	4.17	3.96	4.37	
Transfer rate (%)	59.15	56.41	63.43	59.66
Purity rate (%)	54.58	53.37	56.61	54.85

Table S4. Tumor weight, tumor inhibitory rate, body weight (BW) profile, complete blood count and biochemical profile after treatment with total protoflavones from *M. viridifrons* for 10 days.

	Vehicle control	Positive control	Low dosage	Middle dosage	High dosage
Weigh of tumor (g)	1.65±0.20	0.85±0.12	1.30±0.35	0.88±0.41	0.73±0.28
Tumor inhibitory rate (%)		48.48	21.2	46.66	55.76
BW before treatment (g)	22.0 ± 2.5	21.0 ± 1.6	19.9 ± 0.7	20.5 ± 0.7	21.8 ± 2.2
BW after treatment (g)	30.3 ± 1.9	28.9 ± 1.3	29.5 ± 2.3	28.5 ± 1.8	29.7 ± 0.7
RBC (× 10 ¹² /L)	7.5 ± 0.5	8.4 ± 1.1	7.0 ± 1.5	7.4 ± 1.4	7.3 ± 0.9
WBC (× 10 ⁹ /L)	5.3 ± 0.7	5.4 ± 0.2	5.3 ± 0.8	6.7 ± 0.5	4.4 ± 1.3
PLT (× 10 ⁹ /L)	377.0 ± 141.2	356.0 ± 57.6	538.5 ± 121.1	446.5 ± 76.9	443.0 ± 88.7
HGB (g/L)	116.0 ± 8.6	130.5 ± 5.7	130.5 ± 4.5	134.5 ± 18.2	120.5 ± 10.0
ALT (U/L)	90.0 ± 3.2	88.5 ± 2.5	27.5 ± 1.8	74.0 ± 1.6	57.4 ± 1.4
AST (U/L)	323.0 ± 116.3	314 ± 88.9	269 ± 15.6	256 ± 54.1	175.6 ± 13.1
BUN (mmol/L)	8.3 ± 0.8	7.7 ± 1.0	6.4 ± 0.6	7.3 ± 1.2	9.6 ± 0.4
Cr (μmol/L)	6.7±1.1	9.9 ± 0.4	10.0 ± 1.5	9.8 ± 1.7	7.8 ± 1.1

Data are presented as means ± SD (n=10).

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