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Simultaneous determination and pharmacokinetic studies of dihydromyricetin and myricetin in rat plasma by HPLC-DAD after oral administration of *Ampelopsis grossedentata* decoction

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Abstract

A simple and sensitive high-performance liquid chromatographic method was developed for the simultaneous determination of dihydromyricetin (**1**) and myricetin (**2**) in rat plasma after orally administrating the decoction of *Ampelopsis grossedentata*. Plasma samples were acidified with 0.375% phosphoric acid and extracted with ethyl acetate. Analysis of the extract was performed on reversed-phase C_{18} column with a gradient eluent composed of acetonitrile and 0.04% phosphoric acid. The flow rate was kept at 1 ml/min and the detection wavelength was set at 290 and 370 nm for **1** and **2**, respectively. The calibration curves were linear in the range of 0.247–4.114 μg/ml and 0.150–2.501 μg/ml for **1** and **2**, respectively. The intra-day and inter-day precisions were better than 4.9 and 6.2%, respectively. The limits of detection (LOD) for **1** and **2** in plasma were 21.600 and 52.530 ng/ml, and the limits of quantification (LOQ) were 0.247 and 0.150 μg/ml, respectively. The mean recoveries for **1** and **2** were 92.0 and 93.3%, respectively. The accuracy and precision were well within the acceptable range and R.S.D. of measured rat samples was less than 7.5%. This validated method has been successfully applied in the pharmacokinetics study of dihydromyricetin and myricetin *in vivo* after orally administrating the decoction of *A. grossedentata* to rats.

Keywords: *Ampelopsis grossedentata*; Pharmacokinetics; HPLC-DAD; Dihydromyricetin; Myricetin

1. Introduction

Rattan Tea, the tender stems and leaves of *Ampelopsis grossedentata* (Hand.-Mazz) W.T. Wang, has been widely used as a type of healthy tea product in China. It was reported that Rattan Tea possessed a number of biological functions, including hypoglycemic, antithrombotic, antioxidant, immunostimulating, anti-inflammatory and antibacterial activities [1–4]. The phytochemical investigations showed that dihydromyricetin (**1**) and myricetin (**2**) (Fig. 1) are the two main flavonoids of Rattan Tea. The contents of **1** and **2** were determined to be in the range of 20–30% (w/w) and 1.5–3.0% (w/w) in the dry tender stems and leaves, respectively [5,6], which were considered to be the main active components of Rattan Tea [3,7–12].

Quantification and pharmacokinetics studies on constituents of Traditional Chinese Medicine in plasma are required to offer suitable references in clinical application. Several analytical methods, including HPLC [13–15], LC–MS [16–18], GC–MS [19] and CE (capillary electrophoresis) [20–22], have been developed for the determination of **1** or **2** in the plants, foods and biological samples. There are few reports on determining **1** [23] or **2** [24] in biological sample. However, to our knowledge, there has been no report on the simultaneous determination of **1** and **2** in biological fluids after orally administrating the decoction of Rattan Tea and their pharmacokinetic studies in rats. In this paper, a simple and sensitive HPLC-DAD method for the

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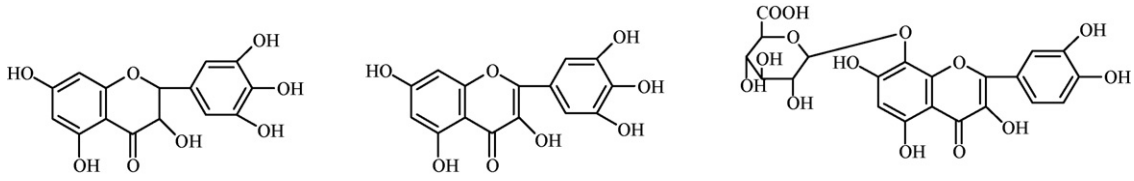

1. dihydromyricetin (DMY) 2. myricetin (MY) 3. hibifolin (HF)

Fig. 1. Structures of dihydromyricetin, myricetin and hibifolin (IS).

simultaneous determination of **1** and **2** in rat plasma was developed. A compound, hibifolin (**3**, HF), was selected as the internal standard (Fig. 1). The method was demonstrated to be successful for application studies on pharmacokinetics of the two compounds.

2. Experimental

2.1. Chemicals and reagents

Rattan Tea was obtained from Chacheng County, Guangxi Zhuang Autonomous Region. Based on plant morphology, it was identified as *A. grossedentata* by Professor Hu-biao Chen, Peking University (Beijing, China), and the voucher specimen was kept in the Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University. The reference standards of dihydromyricetin (**1**) and myricetin (**2**) were separated from *A. grossedentata* and their structures were determined by spectroscopical methods (UV, ^{1}HNMR, ^{13}CNMR, HMQC and HMBC) [25]. The purity was not less than 98% by the external standard method for HPLC analysis. The internal standard, hibifolin (HF > 98% purity), was provided by Dr. Xian-yin Lai, Peking University (Beijing, China). Acetonitrile was of HPLC grade (Fisher, Fair Lawn, NJ). Ultra-pure water was purchased from Wahaha Co., Ltd. (Hangzhou, China). The anticoagulant for the plasma, heparin sodium injection, was purchased from Wanbang Biochemical Pharmaceutical Co. (Jiangsu, China). All other chemical solvents were of analytical grade from Beijing Chemical Factory (Beijing, China). Control plasma used for calibration curve and validation of the assay was obtained from male Sprague–Dawley rats (Laboratory of Animal Center, Peking University Health Science Center, Beijing, China).

2.2. Instrumentation and chromatographic conditions

A LC-10A VP HPLC system (Shimadzu, Kyoto, Japan) consisted of a quaternary pump, a diode-array detector and Class VP for data collection was used. The chromatography was performed on a YMC C_{18} column (5 μm, 250 mm × 4.6 mm I.D.) supplied by YMC Co., Ltd. (Kyoto, Japan) with a Phenomenex RP-C_{18} guard column (California, U.S.A.). The mobile phase consisted of acetonitrile (MeCN, A) and water containing 0.04% (v/v) phosphoric acid (B). A gradient program was used as follows: the initial elution condition was A-B (12:88, v/v), linearly changed to A-B (13:87, v/v) at 8 min, A-B (14:86, v/v) at 22 min, A-B (18:82, v/v) from 30 min to 50 min, A-B (40:60, v/v) at 55 min, A-B (80:20, v/v) at 60 min. The mobile phase was filtered through a 0.45 μm Millipore filter and degassed before use. The flow rate was 1.0 ml/min. The detector wavelengths were set at 290 nm for **1** and IS, 370 nm for **2** and IS, respectively. The injection volume was 20 μl and the column temperature was set at 30 °C.

2.3. Preparation of stock, working solution and internal standard solution

The concentrated stock solutions of **1** and **2** were prepared by dissolving the reference standards in methanol to final concentration of 0.432 mg/ml for **1** and 0.394 mg/ml for **2**. For the assay of plasma samples, working solutions were prepared by appropriate dilution of the stock solution with methanol. The internal standard (**3**) solution was prepared at a final concentration of 84 μg/ml in methanol. All solutions were protected from light and stored at 4 °C.

2.4. Preparation of the decoction of Rattan Tea

Rattan Tea (100 g) was extracted thrice with boiling water, 1 h for each time. Then the solution obtained was concentrated under reduced pressure and lyophilized. The dried powder (31.30 g) was stored at −20 °C and suspended in 0.5% Carboxymethylcellulose Sodium (CMC) to make decoction of Rattan Tea with the concentration of 78.24 mg/ml before use.

2.5. Sample preparations

After orally administrating the decoction of Rattan Tea, the rat plasma was collected. A 400 μl plasma sample was put into a centrifuge tube and acidified with diluted phosphoric acid to pH 2–3. Then 20 μl of internal standard solution (84 μg/ml) was added, and the resulting solution was vortexed vigorously for 3 min, then extracted with 2 ml of ethyl acetate and centrifuged at 537 × *g* for 15 min, where ethyl acetate acted as a deproteinization–extraction agent. The procedure of extraction was performed three times. The organic layer was dried at 35 °C under nitrogen stream. The residue was dissolved in 100 μl methanol, and 20 μl was injected into the HPLC column for analysis. The method of sample preparation was also applied for determination of accuracy, precision and recovery.

2.6. Validation procedure

2.6.1. Linear range, limit of detection (LOD) and limit of quantification (LOQ)

The control plasma samples spiked with **1** and **2** at seven concentrations over the range of 0.247–4.114 μg/ml and 0.150–2.501 μg/ml were prepared as described in Section 2.5. The calibration curves were constructed by plotting the peak area ratios of aimed compounds to IS versus the compound concentrations spiked.

The LOD was determined at the signal height-to-noise of baseline in the ratio of 3:1. The LOQ in plasma was defined as the lowest calibrator that showed precision equal to or better than 20% and accuracy between 80 and 120% of the nominal concentration.

2.6.2. Precision and accuracy

The intra-day precision was determined within one day by analyzing five replicates of each reference standard at concentrations of 0.411, 0.823 and 2.469 μg/ml for DMY and 0.250, 0.500 and 1.501 μg/ml for MY. The inter-day precision was determined on five separate days for each reference standard (three samples of each quality control sample). The intra-day and inter-day precision was defined as R.S.D. and the accuracy was assessed by comparing the measured concentration with its true value.

2.6.3. Extraction recovery

The recoveries of **1** and **2** from plasma were determined at three different levels (low, medium and high) by spiked standards at three concentrations: 0.411, 0.823, 2.469 μg/ml and 0.250, 0.500, 1.501 μg/ml, respectively. The extraction recovery was calculated by comparing mean peak areas of three spiked samples with mean peak areas of the same amounts of unextracted solutions ($n = 3$).

2.6.4. Specificity

Interference from endogenous compounds was investigated by analyzing blank plasma samples. Interference from analytes **1** and **2** was also investigated. 20 μl working solution (including 17.2 μg/ml for **1** and 10.5 μg/ml for **2**) was added into 400 μl control plasma samples.

2.6.5. Stability

The storage stability for **1** and **2** in plasma samples stored at −20 °C for 10 days was studied at three concentrations.

2.7. Application of the method

The utility of the worked-out method was demonstrated under *in vivo* conditions. Male Sprague–Dawley rats were anesthetized using ether before collecting plasma sample. After being fasted for 12 h with free access to water, each rat ($n = 12$) was administered a dose of 1564.8 mg/kg of Rattan Tea decoction. Blood samples were collected from fossa orbitalis vein of the anesthetized rats at 5, 10, 20, 30, 40, 60, 120, 240 and 360 min after dosing. The blood samples were immediately transferred into heparinized tubes and centrifuged for 10 min at 2147 × *g*. The plasma was separated and stored in polypropylene tubes at −20 °C until analysis. For minimizing individual difference, plasma specimens were collected from the first rat for the first 20 min following administration. A second rat was utilized for collection of specimens from 30 min to 360 min. Six rats were utilized for each of the time period. The study protocol was approved by the Animal Care and Use Committee of Peking University Health Science Center.

2.8. Pharmacokinetic data analysis

The pharmacokinetic parameters were determined using the standard non-compartmental method and calculated using Practical Pharmacokinetic Program-Version 87 (3P87, published by Chinese Pharmacological Association, Beijing, China).

3. Results and discussion

3.1. Method specificity

Several substances were tested as internal standards. Among these, hibifolin has been chosen to be the most appropriate in the present analysis because it is stable and does not exist endogenously in plasma. Moreover, in the present study, its retention time was between that of **1** and **2**. In addition, it did not interfere with the matrix of plasma samples and it was well separated from **1** and **2**.

In a previous report [23], analytes were detected by UV detector at a single wavelength. However, the structures of compounds **1** and **2** were flavanonol and flavonol, respectively, showing UV absorption maxima at different wavelengths. The detector wavelengths at 290 nm and 370 nm were chosen for **1** and **2**, respectively. In order to obtain good HPLC chromatograms with a baseline separation of compounds in plasma various HPLC columns were investigated, and the results showed that YMC column was suitable for the analysis.

Since there existed interference ingredients in the plasma, and the polarity of **1** was largely different from that of **2**, several kinds of mobile-phase systems were investigated. Finally, gradient eluent system (acetonitrile–0.04% phosphoric acid; Table 1) was chosen and run time was 60 min. No interference was observed under the assay conditions. The peaks of the analytes in the plasma were identified by comparing their retention times with those of the standards and further confirmed by their on-line

Table 1
The mobile phase used for the HPLC analysis

Time (min)	Acetonitrile (%, vol.)	0.04% Phosphoric acid (%, vol.)
0	12	88
8	13	87
22	14	86
30	18	82
50	18	82
55	40	60
60	80	20

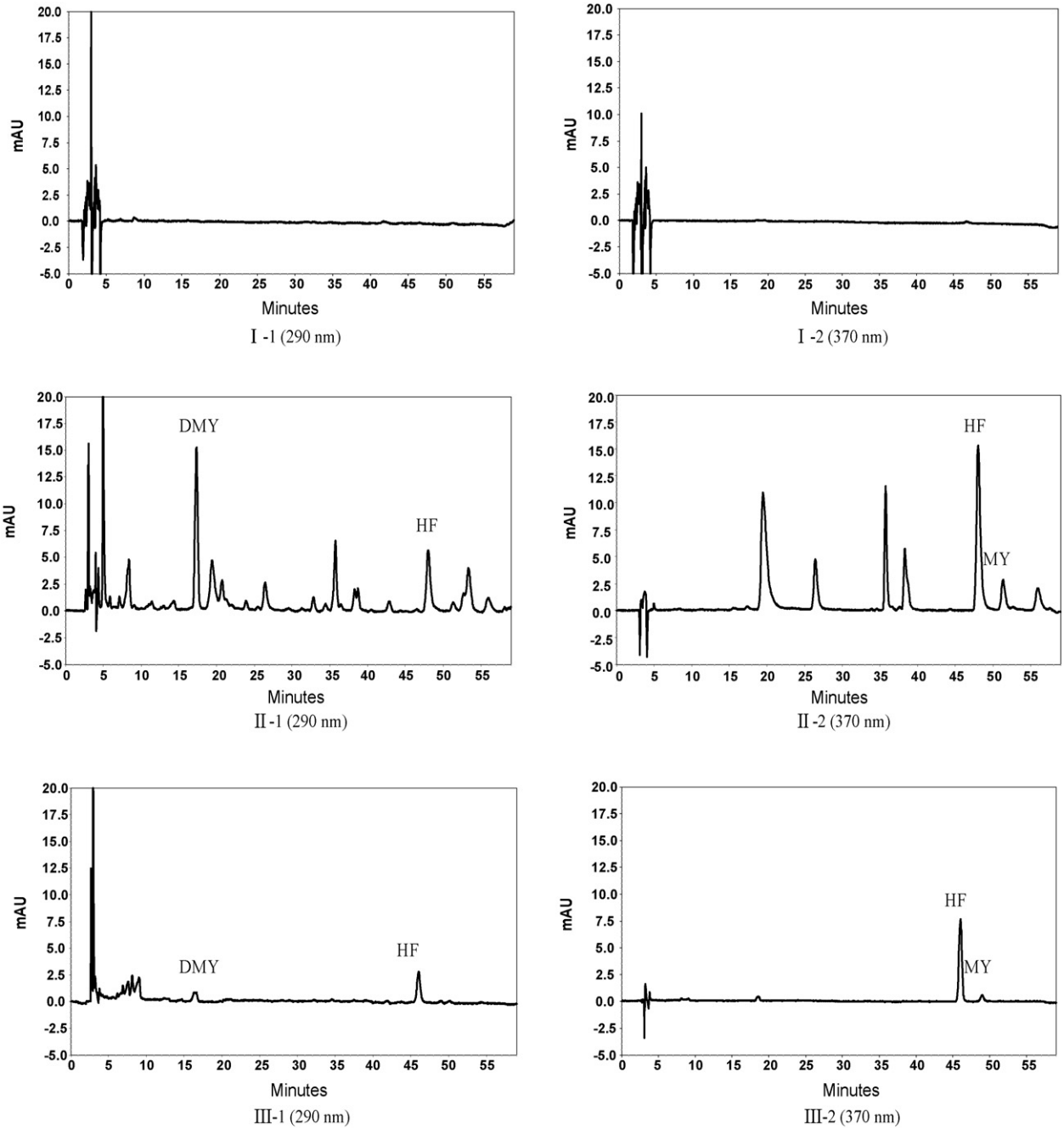

Fig. 2. Typical chromatograms for the determination of **1** and **2** in rat plasma: (I) Blank plasma sample; (II) plasma sample collected at 20 min after oral administration of Rattan Tea decoction; (III) plasma sample spiked with **1**, **2** and IS with concentrations at the lower limit of quantitation. Detection wavelengths are 290 nm (the left chromatogram) and 370 nm (the right chromatogram).

UV–vis spectra. Typical retention times were as follows: DMY, 17.65 min; HF, 48.42 min; MY, 51.77 min. The representative HPLC chromatograms were shown in Fig. 2. Moreover, there were other compounds from the decoction and metabolites.

During the development of the extraction method, both solid-phase extraction (SPE) and liquid–liquid extraction (LLE) were investigated for the sample preparation. However, the results showed that the aimed compounds could not be isolated completely from the proteins and interfering peaks in rat plasma by SPE, but could be isolated by LLE. Therefore liquid–liquid extraction by ethyl acetate was chosen. Finally, pH 2–3 was chosen to obtain good extraction recovery of aimed compounds after several pH values were investigated. The extraction method of Tsuchiya [24] involved solid-phase extraction to retain polyhydroxyflavones from plasma using phenylboric acid cartridges. However, this extraction method is expensive and is not suitable for processing of multiple samples in a limited amount of time for bioequivalence and pharmacokinetic studies. LLE required low volume of organic solvent, and this considerably reduced the sample processing time. The present method is rapid, and extraction using ethyl acetate is simple without any loss of analytes.

Table 2
Intra-day and inter-day precision and accuracy

	Spiked conc. (μg/ml)	Intra-day ($n=6$)			Inter-day ($n=4$)		
		Found conc. (μg/ml)	R.S.D. (%)	Accuracy (%)	Found conc. (μg/ml)	R.S.D. (%)	Accuracy (%)
DMY	0.411	0.427 ± 0.018	4.2	103.9	0.423 ± 0.017	4.0	102.8
	0.823	0.864 ± 0.042	4.9	105.0	0.816 ± 0.047	5.8	99.2
	2.469	2.781 ± 0.098	3.5	112.7	2.655 ± 0.078	3.1	107.6
MY	0.250	0.220 ± 0.005	2.3	88.0	0.222 ± 0.005	2.3	88.8
	0.500	0.499 ± 0.013	2.6	99.7	0.466 ± 0.029	6.2	93.1
	1.501	1.309 ± 0.018	1.4	87.2	1.281 ± 0.033	2.6	85.3

Table 3
Extraction recovery of the method ($n=3$)

Dihydromyricetin				Myricetin			
Spiked conc. (μg/ml)	Recovery (%)	R.S.D. (%)	Average (%)	Spiked conc. (μg/ml)	Recovery (%)	R.S.D. (%)	Average (%)
0.411	86.6 ± 6.5	7.5	92.0	0.250	88.5 ± 5.2	5.9	93.3
0.823	91.7 ± 4.2	4.6		0.500	95.6 ± 3.2	3.4	
2.469	97.8 ± 2.1	2.2		1.501	95.8 ± 2.0	2.0	

3.2. Validation

3.2.1. Linearity and sensitivity

Calibration curves of the test compounds were established by the IS technique following linear regression analysis of plotting plasma concentrations of **1** and **2** (0.247–4.114 μg/ml, 0.150–2.501 μg/ml) against peak area ratio of analytes to IS. The linear regression equation of analytes were $y = 0.8999x + 0.0937$ (r^2=0.9966) and $y = 0.503x - 0.0657$ ($r^2 = 0.9934$), respectively.

The LOD for **1** and **2** in plasma were 21.600 and 52.530 ng/ml, respectively, while the LOQ for **1** and **2** were 0.247 and 0.150 μg/ml, respectively.

3.2.2. Accuracy and precision

The intra-day and inter-day precision and accuracy were shown in Table 2. The intra-day accuracy of **1** and **2** was 103.9–112.7% and 87.2–99.7% with the R.S.D. values less than 4.9% and 2.6%, while the inter-day accuracy was 99.2–107.6% and 85.3–93.1% with the R.S.D. values less than 5.8% and 6.2%, respectively.

3.2.3. Recovery and stability

The mean extraction recoveries and R.S.D. for **1** and **2** from rat plasma were assessed at low, medium and high concentrations. The recoveries of two compounds ranged from 86.6% to 97.8% with R.S.D. no more than 7.5% and from 88.5% to 95.8% with R.S.D. less than 5.9%, respectively. The mean extraction recoveries for **1** and **2** were 92.0% and 93.3%, respectively (Table 3).

The results of storage stability in rat plasma are shown in Table 4. Compounds **1** and **2** were stable in plasma at −20 °C for 10 days.

3.3. Application of the analytical method in pharmacokinetics study

The described method was applied to the analysis of rat plasma after orally administrating the decoction of Rattan Tea. The mean plasma concentration–time profiles of **1** and **2** ($n=6$) are shown in Fig. 3. On the basis of plasma concentrations of **1** and **2** following orally administrating decoction of Rattan Tea, two-compartmental pharmacokinetic parameters are cal-

Table 4
Stability of dihydromyricetin and myricetin ($n=3$)

	Spiked conc. (μg/ml)	Storage stability		
		Concentration (B.S. μg/ml)	Concentration (A.S. μg/ml)	R (%)
DMY	0.411	0.391 ± 0.012	0.341 ± 0.012	87.2
	0.823	0.779 ± 0.009	0.728 ± 0.036	93.5
	2.469	2.590 ± 0.215	2.573 ± 0.019	99.3
MY	0.250	0.272 ± 0.009	0.255 ± 0.006	93.8
	0.500	0.449 ± 0.040	0.428 ± 0.036	95.3
	1.501	1.457 ± 0.080	1.237 ± 0.046	84.9

R, recovered = (after storage/before storage) × 100%; B.S.: before storage; A.S.: after storage.

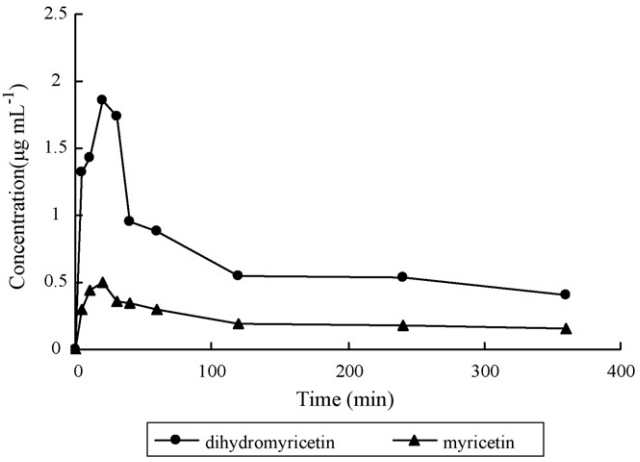

Fig. 3. Mean plasma ($n = 6$) concentration–time curves for **1** and **2** in rat plasma after oral administration of Rattan Tea decoction.

Table 5
Mean pharmacokinetic parameters of dihydromyricetin and myricetin in rat plasma ($n = 6$) after orally administrating decoction of Rattan Tea

Parameter	Estimate (mean ± S.D.)	
	Dihydromyricetin	Myricetin
t_{max} (min)	14.9 ± 0.7	14.7 ± 0.6
C_{max} (μg/ml)	1.7 ± 0.0	0.47 ± 0.01
$AUC_{0-\infty}$ (μg min/ml)	406.3 ± 8.8	301.9 ± 2.3
$t_{1/2}$ (min)	523.9 ± 8.9	978.8 ± 379.3
MRT (min)	140.2 ± 0.1	152.0 ± 0.9

t_{max}, Time to reach maximum concentration; C_{max}: maximum plasma concentration; $AUC_{0-\infty}$, area under the concentration–time curve from zero to infinity; $t_{1/2}$, apparent elimination half-life; MRT, the sum mean absorption and mean residence time.

culated and listed in Table 5. These parameters indicated that both compounds were absorbed and cleared quickly.

4. Conclusion

The present study described a simple and reliable HPLC-DAD with IS method for the determination of two flavonoids in blood samples. This is the first validated HPLC method for analysis of dihydromyricetin and myricetin in rat plasma, which has good recovery, sensitivity and reproducibility. This method has been successfully applied to the pharmacokinetic study of **1** and **2** following orally administrating decoction of Rattan Tea. The pharmacokinetic results are useful for the further studies on the clinical applications of the Rattan Tea.

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