Cryptochlorogenic acid and its metabolites ameliorate myocardial hypertrophy through HIF1α related pathway

S1 Experimental

S1.1 Chemicals and reagents

The reference standard 4-CQA was purchased from Push Bio-technology Co. (Chengdu, China). Caffeic acid and puerarin were purchased from Shanghai Yuanye Bio-technology Co. (Shanghai, China), 4-*O*-feruloylquinic acid was purchased from ChemFaces (Wuhan, China). The purity of all reference substances is greater than 98%. HPLC grade acetonitrile, methanol and formic acid (FA) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). All the other chemicals of analytical grade are available at the work station, Beijing Chemical Works (Beijing, China). Deionized water used throughout the experiment was purified by a Milli-Q Gradient Å 10 System (Millipore, Billerica, MA, USA). Oasis HLB C_{18}-low solid-phase extraction cartridges (500 mg/3 mL, 60 μm, 70 Å) for the pretreatment of biological samples were supplied by Waters Scientific Co. (Milford, USA).

S1.2 Instrumentation

Analysis was carried out on ACQUITY UHPLC system connected with Xevo TQ-S-Micro triple quadrupole mass spectrometer equipped with an electrospray ionization source (Waters Corporation, Milford, MA, USA).

The separation of analytes was performed on a reversed-phase Waters ACQUITY HSS T3 column (2.1×100 mm, 1.7 μm; Waters Corporation, Milford, MA, USA) with a column oven at 35℃. The mobile phase for HPLC consisted of 0.1% FA aqueous solution (A) and acetonitrile (B) with a flow rate of 0.3 mL/min. A gradient program was used as follows: 0-1.5 min, 5%-13%B; 1.5-5.5 min, 13%B; 5.5-6.5 min, 13%-20%B; 6.5-7 min, 20%-95% B; 7-7.5min, 95%-5% B; 7.5-8 min, 5% B. MS data was acquired with the negative ion mode by multiple reaction monitoring (MRM) under the following conditions: flow rates of desolvation gas of 1000 L/Hr, desolvation temperature 500 ℃, source temperature of 150 °C, a capillary voltage of 2.5 KV. All the raw data were processed using Masslynx V4.2. Mass parameters were shown in

Table S1.

Table S1. Mass parameters of multiple reaction monitoring (MRM) for detecting metabolites of 4-CQA and puerarin (internal standard) based on LC-MS/MS

Compound	Precursor ion [M-H]$^-$	Product ion [M-H]$^-$	dwell time (s)	Cone voltage (V)	Collision energy (eV)
4-CQA	353.13	172.89	0.045	-2	-14
Caffeic acid	178.82	134.84	0.045	-2	-16
4-*O*-feruloylquinic acid	367.14	172.90	0.045	-2	-14
puerarin	415.08	294.99	0.045	-70	-16

S1.3 Preparation of standard solutions

The internal standard puerarin (IS) powders was dissolved in methanol at a final concentration of 50 ng/mL as working solution. Working standard solutions of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid were prepared by serial dilution of primary stock solutions with methanol to obtain concentrations of 25~25000 ng/mL, 5~5000 ng/mL, 5~5000 ng/mL, respectively. All solutions were kept at 4 ℃ in refrigerator and were brought to room temperature prior to use.

S1.4 Sample preparation

The plasma samples were thawed to 4 ℃ before processing. An aliquot of 100 μL plasma sample was transferred to an eppendorf tube, 100 μL IS (50 ng/mL) and 100 μL of hydrochloric acid (1M) was added to vortex-mixed for 30 sec. Then, 800 μL ethyl acetate solution was added to precipitate the plasma proteins and vortex-mixed for 5 min. The samples were centrifuged at 12,000 rpm for 10 min. The supernatant was transferred and evaporated to dryness under gentle stream of nitrogen at room temperature. The residue was reconstituted in 50 μL of initial mobile phase and 5 μL aliquot was injected for analysis.

S1.5 Analytical method validation

The validation of this method was carried out with regard to specificity, linearity, precision, accuracy, stability, recovery and matrix effect.

S1.5.1 Specificity

The specificity was evaluated by comparing the chromatograms of blank plasma, blank plasma spiked with IS and standard mixture, and rat plasma sample obtained 1 h after intragastric administration of 4-CQA.

S1.5.2 Linearity and LLOD

An aliquot of 80 μL blank plasma sample was transferred to an eppendorf tube, 20 μL different concentration standard mixture was added to vortex-mixed respectively. Calibration curve was conducted by linear regression of the peak area ratio (y) of targets to internal standard, versus spiked concentration (x) in ng/mL, respectively. In a Weighted regression procedure, the weight factor was set to $1/x^2$. The lower limit of quantification (LLOQ) were defined as the lowest concentration standard in the calibration curve with the signal-to-noise ratio over 10 and was analyzed with accuracy within ±20% and a precision ≤20%.

S1.5.3 Precision and accuracy

The precision and accuracy were determined by analyzing Quality control (QC) samples for three concentration levels. Each level was analyzed six times on the same day for intra-day precision and accuracy, and on three consecutive days for inter-day precision and accuracy. Precision was expressed as relative standard deviation (RSD) at each concentration level and accuracy was calculated by relative error (RE). For accuracy, the criterion for the acceptability of data should not exceed 15% for each concentration level. Similarly, the value for precision also should not deviate by ±15% from the nominal concentration.

S1.5.4 Recovery and Matrix effect

The recovery was determined by comparing peak area of rat plasma samples spiked with three different concentrations of standard mixture before and after extraction. Each time, the concentrations of added standards reached a QC level. The matrix effect was evaluated by comparing the peak area of the analytes that were spiked into the post-precipitation matrix with those of standard solutions in the absence of matrix for three QC levels. Six replicates for each QC level were performed.

S1.5.5. Stability

The stability was performed by triplicate assay at three QC concentration levels under various storage or handing conditions, including stability of plasma samples at -80℃ for one month (long term), stability of plasma samples at room temperature for 4 h (short-term), stability after three freeze-thaw cycles storage at -20℃ for 48 h. It was evaluated by comparing the measured concentration with those of the respective freshly prepared QC samples.

S2. Results

S2.1 Specificity

The specificity was evaluated by comparing the chromatograms of blank plasma, blank plasma spiked with IS and standard mixture, and rat plasma sample obtained 1 h after intragastric administration of 4-CQA. Representative chromatograms were presented in **Fig. S1**. It can be seen that 4-CQA, caffeic acid, 4-*O*-feruloylquinic acid and IS were sufficiently separated and no interfering peak was observed at the retention times of these compounds.

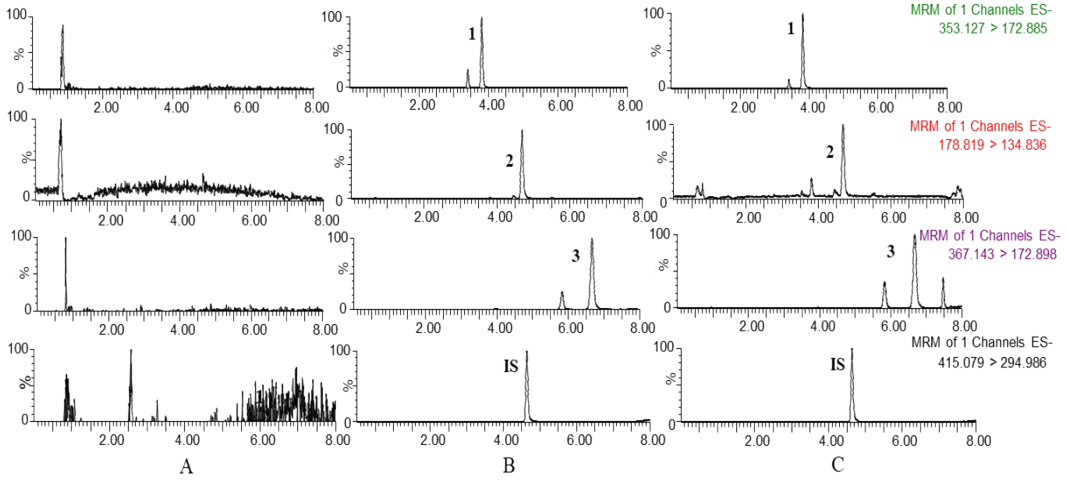

Fig. S1 Representative chromatograms of blank plasma (A), blank plasma spiked with IS and standard mixture (B) and rat plasma sample obtained 1 h after intragastric administration of 4-CQA (1:4-CQA; 2: caffeic acid; 3:4-*O*-feruloylquinic acid)

S2.2 Linearity and LLOQ

After processing the data, the regression curve of each component presented high linearity with a correlation coefficient (r) larger than 0.9986. The linear regression equation of calibration curves for 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid was $y = 0.0105\ x\text{-}0.2224$, $y = 0.0453\ x\ \text{-}0.1655$, $y = 0.0139\ x\text{+}0.5963$, respectively (shown

in **Table S2**). In addition, the LLOQs of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid were 5 ng/mL, 1 ng/mL and 1 ng/mL with the signal-to-noise ratio over 10, respectively, which were sufficient for pharmacokinetic study of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid following an intragastric administration of 4-CQA to rats.

S2.3 Precision and accuracy

The precision and accuracy results at low, medium and high concentrations of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid in rat plasma are demonstrated in **Table S3**. The results showed that precisions and accuracies at three concentration levels (6 replicates) were within 13.72% and 88.75%~110.50%, respectively. The high precision and accuracy indicated that the present method was reliable and reproducible for the quantitative analysis of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid in plasma sample.

S2.4 Recovery and matrix effect

Table S4 summarizes the matrix effect and recovery of measured 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid in plasma. The results showed that recovery of three compounds at three concentration levels were 85.13%~97.46% and the values of RSD were 2.91%~13.45%. The values of matrix effects for all targets were close to 1 and the values of RSD were 1.49%~11.36%, indicating that the matrix effect could be ignored and the present method was sufficient for the quantitative analysis of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid in plasma sample.

S2.5 Stability

The stability results of short-term (at room temperature for 4 h), three freeze-thaw (at -20℃ for 48 h), and long-term storage (at -80℃ for one month) at three QC levels were summarized in **Table S5**, which were sufficient for pharmacokinetic study of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid following an intragastric administration of 4-CQA to rats.

Table S2. Linearity and regression equation of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid

Compound	Linearity and range (ng/mL)	Correlation coefficient (*r*)	Regression equation	Quantization limit (ng/mL)
4-CQA	5~5000	0.9994	$y = 0.0105\ x - 0.2224$	5
caffeic acid	1~1000	0.9995	$y = 0.0453\ x - 0.1655$	1
4-*O*-feruloylquinic acid	1~1000	0.9986	$y = 0.0139\ x + 0.5963$	1

Table S3. Precision and accuracy of of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid in rat plasma

Compound	Concentration (ng/mL)	Intraday (n = 6)			Interday (n = 6)		
		Measured value (ng/mL)	Accuracy (%)	Precision (%)	Measured value (ng/mL)	Accuracy (%)	Precision (%)
4-CQA	10	10.07	100.70	13.72	9.15	91.50	10.3
	500	451.39	90.28	5.14	457.93	91.59	5.73
	4000	3675.26	91.88	5.58	3549.87	88.75	3.16
caffeic acid	2	2.07	103.50	5.86	1.97	98.50	5.15
	100	100.53	100.53	2.71	106.13	106.13	4.68
	800	806.06	100.76	3.22	800.20	100.03	1.84
4-*O*-feruloylquinic acid	2	2.21	110.50	6.03	2.00	100.00	9.66
	100	100.21	100.21	8.78	99.27	99.27	0.99
	800	761.92	95.24	4.00	777.46	97.18	3.45

Table S4. Recovery and matrix effect of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid in rat plasma

Compound	Concentration (ng/mL)	Recovery		Matrix effect	
		Mean (%)	RSD (%)	Mean (%)	RSD (%)
4-CQA	10	87.02	13.45	86.92	7.70
	500	90.43	3.12	106.06	11.36
	4000	87.27	8.31	85.58	3.95
Caffeic acid	2	85.45	7.03	94.47	2.15
	100	97.46	7.71	100.19	9.83
	800	89.78	6.18	97.02	4.13
4-*O*-feruloylquinic acid	2	89.35	3.78	91.33	9.18
	100	85.13	2.91	96.07	1.49
	800	86.64	3.76	87.28	3.73

Table S5. Stability of of 4-CQA, caffeic acid and 4-*O*-feruloylquinic acid in rat plasma

Compound	Concentration (ng/mL)	Short-term		Freeze-thaw		Long-term	
		Measured value (ng/mL)	RSD (%)	Measured value (ng/mL)	RSD (%)	Measured value (ng/mL)	RSD (%)
4-CQA	10	9.64	7.38	9.34	5.91	9.25	8.87
	500	429.71	5.18	436.14	3.66	461.62	2.53
	4000	3591.92	5.58	3573.84	5.67	3518.31	8.78
caffeic acid	2	1.92	7.21	1.81	8.85	1.98	2.32
	100	105.08	1.79	105.59	2.65	101.47	2.60
	800	790.52	3.50	773.42	6.84	803.78	1.03
4-*O*-feruloylquinic acid	2	1.92	13.97	1.78	10.47	1.74	4.23
	100	86.96	3.39	98.71	2.21	95.09	10.50
	800	774.76	4.10	778.11	7.50	764.60	6.58