SUPPLEMENTARY MATERIAL

Determination and Comparison of Alkaloids and Triterpenes among Tissues after Oral Administration of Crude and Processed *Phellodendri Chinensis Cortex* by UPLC-QqQ-MS

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Abstract: *Phellodendri Chinensis Cortex* is widely used in the clinic of traditional Chinese medicine. In order to enlarge the range of application, it is necessary to processed with honey, salt-water, and rice-wine, respectively. We hope to elucidate the connotation of processing, an UPLC-QqQ-MS method was used for determination and comparison the tissue distribution of alkaloids and triterpenes after oral administration water-extracts of crude and processed products. The results showed that the berberine, phellodendrine, magnoflorine, limonin, and obacunone in crude and processed products were distributed in all tissues, especially in the small intestine and stomach. In this study, we can provide a scientific basis for explaining the processing connotation of *Phellodendri Chinensis Cortex* processed with salt-water and rice-wine, respectively.

Key words: *Phellodendri Chinensis Cortex*, Alkaloids, Triterpenes, Tissues, Processing, UPLC-QqQ-MS

Experimental

Materials and reagents

CHB was purchased from GAP Planting Base, Ya’an, Sichuan Province, China. It was identified and authenticated by Professor ZAI Yan-Jun according to the standards of Chinese Pharmacopoeia 2015. Department of Identification of Chinese medicine, Liaoning University of TCM, Dalian, China. The processed CHB comes from the same batch CHB. The voucher specimens were deposited in the Chinese Materia Medica Processing Engineering Center of Liaoning Province, Liaoning University of TCM. Standard substances (purity, 98%) of berberine, phellodendrine, magnoflorine, limonin, and obacunone were purchased from Must company (Sichuan, China). The Internal standard substance (IS) called carbamazepine (purity, 98%) was purchased from the National Institute for the control of Biological and Pharmaceutical Drugs (Beijing, China). Ultrapure water was produced by Milli-Q system (18.2 MΩ, Millipore, Billerica, USA). MS-grade acetonitrile, methanol, and HPLC-grade formic acid were purchased from Merck KGaA (Darmstadt, Germany). Brand Tower rice wine was purchased from Zhejiang Brand Tower Shaoxing Wine Co., Ltd. (Zhejiang, China). Pure natural honey was purchased from Shanghai Beisheng Biotechnology Co., Ltd. (Shanghai, China). Lodinefree salt was purchased from Dalian Salt Industry Co., Ltd. (Dalian, China).

UPLC-QqQ-MS condition

Chromatographic analysis was performed in a Waters ACQUITY H-CLASS UPLC system (Waters Corporation, Milford, MA, USA), Using an ACQUITY UPLC® BEH C18 column (50 mm × 2.1 mm, 1.7 μm, Waters). The mobile phase was consisted of (A) acetonitrile containing 0.1% formic acid and (B) water containing 0.1% formic acid, and the best elution conditions were as follows: 15% to 100% A (0−7 min), 100% to 100% A (7−8 min), 100% to 15% A (8−8.01 min), 15% to 15% A (8.01−10 min). The flow rate was set at 0.40 mL·min⁻¹. The temperature of column and auto-sampler room were set at 30 and 8 °C, respectively. The injection volume was 10 μL (Liu, 2015).

Mass spectrometry analysis was performed with a Waters XEVO TQD MS (Waters) with an electrospray ionization (ESI) source in positive ion mode. The desolvation gas (N2) flow rate was set at 900 L·h⁻¹ with a temperature of 450 °C, the source temperature was set at 150 °C, and the cone gas was set at 50 L·h⁻¹. The capillary and cone voltages were set at 3000 and 50 V, respectively. Collision energy was optimized based on the standards. Helium was used as the collision gas for collision-induced dissociation. Quantification was carried out using the multiple reactions monitoring (MRM) mode (Li, 2016; Tian, 2014). The mass spectrometric parameters

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of carbamazepine, berberine, phellodendrine, magnoflorine, limonin, and obacunone are shown in Table S1. The adduct formation of parent ion is \([M + H]^+\).

Table S1. The mass spectrometric parameters of carbamazepine, berberine, phellodendrine, magnoflorine, limonin, and obacunone.

| No. | \(t_0/\text{min}\) | Compound            | Parent ion(m/z) | Daughter ion(m/z) | Cone voltage (V) | Collision energy(V) |
|-----|-----------------|---------------------|-----------------|-------------------|------------------|---------------------|
| 1   | 2.95            | Carbamazepine       | 236.97          | 178.93            | 42.0             | 34.0                |
| 2   | 2.47            | Berberine           | 335.95          | 320.22            | 62.0             | 28.0                |
| 3   | 0.82            | Phellodendrine      | 342.01          | 191.99            | 46.0             | 24.0                |
| 4   | 0.99            | Magnoflorine        | 342.01          | 264.99            | 46.0             | 22.0                |
| 5   | 3.68            | Limonin             | 471.03          | 94.88             | 44.0             | 24.0                |
| 6   | 4.29            | Obacunone           | 455.03          | 160.99            | 40.0             | 44.0                |

Preparation of Phellodendri Chinensis Cortex solution

Taked the appropriate amount of CHB in the casserole, soaked with 10 times the volume of distilled water for 30 min, and then decocted for 60 min and percolated. The residue was redissolved with 8 times the volume of distilled water to decocted for 60 min and percolated again, and combined two filtrates. The final concentration of CHB solution was 1 g/mL. The sample was stored in dry and dark place before use. Respectively, the processed CHB with rice-wine, salt-water and honey products solution were prepared with same method.

Animals

Healthy cleaning grade Sprague-Dawley (SD) rats (male, 190 g ± 10 g, 6-8 weeks old) were purchased from the Animal Center of Benxi Chang Sheng Biotechnology Co. Ltd., (certificate number: SCXK 2010-0001, Benxi, China) and conventionally raised a week before the experiment. The rats were maintained in an air-conditioned animal quarter at a temperature of 22 °C ± 2 °C, humidity of 50% ±10%, and 12 h light/12 h dark cycle. Rats were deprived of food overnight before the experiment but were allowed free access to water. All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals (Liu, 2017; Yuan, 2017).

Drug administration and tissue sampling

For tissue sample, 78 rats were divided into thirteen groups (n = 6 per group) randomly. Rats were oral administration crude, rice-wine processed CHB, salt-water processed CHB, and honey processed CHB water extract at a single dose of 8 g/kg, respectively. Heart, liver, spleen, lung, kidney, stomach, small intestine, and large intestine were collected at 30, 60, 180 min. Tissue samples were weighed rapidly, rinsed with physiological saline to remove the blood or content, blotted on filter paper, and then stored at −20 °C until use (Liu, 2017; Yuan, 2017).

Preparation of tissue sample

Each of the tissue samples was thawed and accurately weighed 0.2 g into an Eppendorf tube, and acquired tissue homogenate with 2 mL of ice-cold physiological saline under homogenate (homogenate 12 s/time, gap 20 s, 3–5 times). In order to eliminate interference of the protein, added 20 μL IS and 2 mL of methanol in 1 mL of tissue homogenate by vortexing for 2 min, and centrifuging at 10 000 r/min for 5 min. The supernatant was transferred into an Eppendorf tube, and evaporated to dryness with nitrogen. The residue was reconstituted in 100 μL methanol, vortexed for 20 s, and centrifuged at 12 000 r/min for 15 min. The 10 μL supernatant was injected onto the UPLC-QqQ-MS system for analysis (Liu, 2017; Yuan, 2017).

Method validation

Specificity

All of the blank tissue homogenates were prepared and analyzed to ensure no interfering peaks. And for the sake of evaluating the method selectivity, to compared the chromatograms of tissue homogenate spiked with the IS and
analytes, and tissue homogenate after an orally dose. There was no interference endogenous components signal detected at the corresponding retention time positions. So, the method has good specificity (Figure S1).

**Calibration curves**

The peak area of target compounds to IS vs. the nominal concentrations of the calibration standards by linear regression were used for calibration curves. There were five concentration levels in the procedure of generating the calibration curves of berberine, phellodendrine, magnoflorine, limonin, and obacunone in homogenates. The calibration curves show a good linear over the concentration in the range of 0.003 μg/mL to 0.300 μg/mL in tissue homogenates of berberine, phellodendrine, magnoflorine, limonin, and obacunone. The values of correlation coefficient r were over 0.9900.

**Recovery and stability**

The extraction recoveries of berberine, phellodendrine, magnoflorine, limonin, and obacunone were achieved by adding standards at three concentration levels to the tissue samples, and then compared the peak areas of the samples with those of the spike-post-extracted samples. Short-term stability was evaluated by assaying samples at three concentration levels, which were stayed at 25°C for 24 h. Long-term stability was assessed by assaying samples at three concentration levels, which were stored at -20°C for 30-day. The freeze-thaw stability of samples was studied on three concentration levels, which were kept at -20 to 25°C and operated in the same way for three freeze-thaw cycles. The extraction recoveries of berberine, phellodendrine, magnoflorine, limonin, and obacunone ranged from 78.4% to 112.8% in tissue samples, indicated that the method can provide reliable data. The RSD values of the stability results under the three conditions were less than 10%, which demonstrated that berberine, phellodendrine, magnoflorine, limonin, and obacunone were stable under the three conditions in tissue samples, indicated that there was no significant sample loss.

**Precision and accuracy**

The intraday precision and accuracy of the determination method were evaluated by examining the samples in three different concentration levels at the same day. And the interday precision and accuracy were assessed by using the same levels of the standard solutions on three consecutive days. The intra-and inter-day precision were expressed as the RSD, and the accuracy was expressed as RE. The RSD values of intraday and interday precision of berberine, phellodendrine, magnoflorine, limonin, and obacunone were less than 5%, and the RE values of accuracy were within ± 10%. So the accuracy and precision of the method were suitable for the determination analysis of the tissue samples.

**Data analyses**

UPLC-QqQ-MS system was applied to detect and determinate the berberine, phellodendrine, magnoflorine, limonin, and obacunone in different tissue samples. The calculated and identified of the parent and daughter ions of the compounds were using the MassLynx V4.1 software (Waters).
Figure S1. (A) Chromatograms of blank tissue homogenate. (B) Blank tissue homogenate with carbamazepine, berberine, phellodendrine, magnoflorine, limonin, and obacunone. (C) Stomach sample (3 h) after oral administration water-extract of crude.
Figure S2. The concentration-time plot of the berberine in the different tissues of the rats.

Figure S3. The concentration-time plot of the phellodendrine in the different tissues of the rats.

Figure S4. The concentration-time plot of the magnoflorine in the different tissues of the rats.
Figure S5. The concentration-time plot of the limonin in the different tissues of the rats.

Figure S6. The concentration-time plot of the obacunone in the different tissues of the rats.

The tissue concentrations of berberine, phellodendrine, magnoflorine, limonin, and obacunone determined at 30, 60, and 180 min after oral administration crude and processed CHB at a dose of 8 g/kg are shown in Tables S2-S6.

Table S2. The berberine concentration in the different tissues of the rats. (n=6)

| Tissue    | Time | Crude   | HCHB | SCHB | RCHB |
|-----------|------|---------|------|------|------|
| Liver     | 30   | 104.4217| 410.8271| 65.5768| 20.2464|
|           | 60   | 131.4370| 153.8332| 74.5746| 107.0698|
|           | 180  | 106.3134| 194.0218| 68.6355| 16.8283|
| Heart     | 30   | 66.2763 | 400.6280| 71.3424| 24.1958|
|           | 60   | 114.5885| 206.4429| 58.6212| 33.7793|
|           | 180  | 72.4911 | 124.7289| 42.8373| 14.6610|
| Spleen    | 30   | 103.4190| 310.6363| 66.1172| 36.5853|
|           | 60   | 131.1319| 103.4079| 59.7792| 18.1188|
|           | 180  | 117.3616| 124.0953| 40.9370| 19.8129|
| Lung      | 30   | 149.0348| 467.2259| 54.9189| 23.2697|
|           | 60   | 112.0110| 245.9631| 53.2644| 23.3764|
|           | 180  | 54.1999 | 86.9260| 38.1014| 12.1902|
| Kidney    | 30   | 58.7610 | 324.2862| 37.4777| 30.4380|
|           | 60   | 95.6097 | 211.7687| 50.1746| 55.1813|
|           | 180  | 130.5615| 84.9040| 37.6781| 22.7218|
| Large intestine | 30 | 139.5100| 262.6057| 205.2503| 73.7037|
|           | 60   | 178.0376| 537.8791| 103.1890| 63.4292|
|           | 180  | 181.7229| 330.1120| 94.5199| 25.4039|
### Table S3. The phellodendrine concentration in the different tissues of the rats. (n=6).

| Tissue         | Time (min) | Concentration (ng/g) | Crude | HCHB | SCHB | RCHB |
|----------------|------------|----------------------|-------|------|------|------|
| Liver          | 30         | 3.9528               | 0.4588| 3.7645| 0.4346|
|                | 60         | 10.4445              | 3.4651| 4.9373| 3.9501|
|                | 180        | 30.6734              | 0.7784| 2.7600| 0.3438|
| Heart          | 30         | 3.1798               | 8.6438| 2.6687| 6.9224|
|                | 60         | 15.9055              | 17.2692| 4.1238| 2.1765|
|                | 180        | 6.7920               | 8.1709| 2.2295| 0.6410|
| Spleen         | 30         | 21.1106              | 75.1370| 2.0425| 2.1317|
|                | 60         | 8.7969               | 5.4098| 4.6859| 1.0287|
|                | 180        | 10.3843              | 7.2958| 1.1526| 0.8916|
| Lung           | 30         | 34.5288              | 71.6405| 7.6193| 1.2320|
|                | 60         | 8.0182               | 20.7293| 3.0874| 1.0408|
|                | 180        | 3.5037               | 5.2904| 1.1630| 0.5574|
| Kidney         | 30         | 11.0357              | 27.8661| 1.1235| 1.3093|
|                | 60         | 10.8341              | 19.1161| 2.3762| 0.8259|
|                | 180        | 8.8914               | 4.6653| 1.3782| 0.4741|
| Large intestine| 30         | 16.0711              | 41.4926| 2.9662| 5.5344|
|                | 60         | 25.2001              | 42.0363| 6.4893| 3.1139|
|                | 180        | 16.9170              | 39.2298| 14.9615| 1.1904|
| Small intestine| 30         | 105.9931             | 419.4512| 24.8817| 22.3776|
|                | 60         | 145.3174             | 197.6970| 82.8009| 43.7448|
|                | 180        | 101.6210             | 108.3339| 56.5646| 12.9879|
| Stomach        | 30         | 228.8193             | 355.9423| 24.9423| 26.1392|
|                | 60         | 136.4847             | 187.7474| 32.9673| 22.6369|
|                | 180        | 37.7297              | 63.5049| 21.7764| 7.1977|

### Table S4. The magnoflorine concentration in the different tissues of the rats. (n=6)

| Tissue         | Time (min) | Concentration (ng/g) |
|----------------|------------|----------------------|
| Liver          | 30         | 646.3930             |
|                | 60         | 1487.4400            |
|                | 180        | 456.8612             |
| Stomach        | 30         | 1316.2220            |
|                | 60         | 690.2532             |
|                | 180        | 370.0151             |

3

4
| Tissue      | Time | Crude     | HCHB     | SCHB     | RCHB     |
|-------------|------|-----------|----------|----------|----------|
| Liver       | 30   | 6.7436    | 0.2257   | 1.9362   | 0.1841   |
|             | 60   | 5.3818    | 0.3672   | 2.1084   | 0.4025   |
|             | 180  | 2.9446    | 0.1585   | 1.2076   | 0.1054   |
| Heart       | 30   | 2.8608    | 4.7125   | 0.9356   | 1.0521   |
|             | 60   | 6.8530    | 5.6498   | 1.1173   | 0.2663   |
|             | 180  | 2.9741    | 1.1619   | 0.5671   | 0.1383   |
| Spleen      | 30   | 15.0849   | 10.6224  | 0.5113   | 0.3692   |
|             | 60   | 3.7940    | 2.0092   | 0.5700   | 0.0903   |
|             | 180  | 0.8210    | 2.1724   | 0.2062   | 0.1096   |
| Lung        | 30   | 12.6188   | 30.4378  | 0.4495   | 0.1651   |
|             | 60   | 3.2977    | 8.1650   | 0.6459   | 0.1750   |
|             | 180  | 1.0400    | 1.8676   | 0.2923   | 0.1107   |
| Kidney      | 30   | 1.0929    | 14.2844  | 0.4370   | 0.2165   |
|             | 60   | 6.4071    | 8.7009   | 0.5594   | 0.3229   |
|             | 180  | 4.4833    | 1.8638   | 0.3352   | 0.1225   |
| Large intestine | 30   | 127.7029  | 281.5267 | 14.8977  | 5.2472   |
|             | 60   | 90.0489   | 107.5155 | 34.9955  | 6.0131   |
|             | 180  | 43.6132   | 70.5610  | 29.8930  | 1.9109   |
| Stomach     | 30   | 159.0166  | 110.4691 | 8.9960   | 5.2296   |
|             | 60   | 86.7040   | 94.7082  | 18.2441  | 2.6783   |
|             | 180  | 14.7579   | 25.2223  | 6.5716   | 0.6283   |

Table S5. The limonin concentration in the different tissues of the rats. (n=6)
| Time | Crude | HCHB | SCHB | RCHB |
|------|-------|------|------|------|
| Liver |
| 30   | 1.8966 | 20.4280 | 11.0936 | 2.0839 |
| 60   | 3.9946 | 2.2800 | 2.6868 | 3.1820 |
| 180  | 1.6310 | 9.5893 | 1.0093 | 0.6094 |
| Heart |
| 30   | 0.5837 | 2.2434 | 2.2036 | 3.2837 |
| 60   | 0.7719 | 2.4813 | 1.1360 | 0.7783 |
| 180  | 0.9479 | 3.8669 | 1.0351 | 0.3201 |
| Spleen |
| 30   | 3.9511 | 7.8609 | 2.5865 | 1.3970 |
| 60   | 1.4869 | 0.7939 | 2.1110 | 0.7803 |
| 180  | 2.0918 | 4.2368 | 0.8526 | 0.7139 |
| Lung |
| 30   | 1.5055 | 3.2933 | 2.3652 | 0.8173 |
| 60   | 0.6675 | 1.0908 | 0.9441 | 0.3647 |
| 180  | 0.4701 | 1.4002 | 0.5688 | 0.3354 |
| Kidney |
| 30   | 0.4893 | 6.8524 | 1.6669 | 1.4845 |
| 60   | 0.7547 | 1.2624 | 1.0130 | 0.4669 |
| 180  | 1.1669 | 1.5638 | 0.3641 | 0.4149 |
| Large |
| 30   | 3.2011 | 6.8514 | 11.6589 | 2.8695 |
|        |    |     |     |     |
|--------|----|-----|-----|-----|
| intestine | 60 | 1.2387 | 3.6317 | 4.3915 | 1.1946 |
|         | 180 | 1.1030 | 2.2733 | 2.7534 | 0.8055 |
| Small   | 30 | 6.3194 | 33.2384 | 14.7710 | 2.4352 |
| intestine | 60 | 3.4546 | 18.3496 | 4.0921 | 1.9503 |
|         | 180 | 2.5101 | 9.0176 | 4.0594 | 0.9898 |
| Stomach | 30 | 144.9751 | 113.3519 | 95.9906 | 50.0544 |
|         | 60 | 47.7622 | 82.6591 | 96.2987 | 32.1335 |
|         | 180 | 15.9948 | 33.1298 | 7.2015 | 14.9240 |

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