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Chemical fingerprint and metabolic profile analysis of *Citrus reticulate 'Chachi'* decoction by HPLC-PDA-IT-MS^n and HPLC-Quadrupole-Orbitrap-MS method

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ABSTRACT

A method incorporating HPLC-PDA-IT-MS^n with HPLC-Quadrupole-Orbitrap-MS was developed for the investigation of chemical fingerprint of *Citrus reticulate 'Chachi'* decoction (CRCD) and metabolic profile of SD rat plasma sample after oral administration of CRCD (1.5 g herb/kg). A total of 27 chemical constituents of CRCD were identified from their MW, UV spectra, MS^n data and retention behavior by comparing the results with those of the reference standards or literature. And 43 compounds were detected in dosed SD rat plasma samples, including 9 prototypes which were identified as hesperetin, isosinensetin, sinensetin, tetramethyl-*O*-isoscutellarein, nobiletin, tetramethyl-*O*-scutellarein, HMF (3,5,6,7,8,3′,4′-heptamethoxyflavone), tangeretin and 5-demethylnobiletin and 34 metabolites underwent metabolic process of demethylation, glucuronide conjugation, sulfate conjugation or mixed modes. This is the first research for the metabolic profile of CRCD in SD rats, which could lay a foundation for the further studies of CRC or its formulation.

1. Introduction

Pericarpium Citri Reticulatae (PCR; Chenpi in Chinese), the dried ripe pericarp of *Citrus reticulata Blanco* or its cultivars, is widely used in traditional Chinese medicine for its low toxicity and various pharmacological activities [1] and officially listed in the Chinese Pharmacopoeia [2]. Traditional Chinese medicine (TCM) theory indicates that it has the efficacy of strengthening spleen and liver, dispersing stagnation, eliminating dampness and resolving phlegm [2,3]. Among the main Chenpi cultivars, the dried ripe pericarp of *Citri reticulate 'Chachi'* which produced in Xinhui of Guangdong Province in China (CRC, Guangchenpi in Chinese) is viewed as a genuine medicinal material. Pharmacological research has demonstrated that CRC exhibits significant anti-inflammatory [4], antioxidant [5,6], antibacterial [7] and anticancer [8] functions. In China, it has been used for centuries as a remedy to treat indigestion and cough and to improve inflammatory syndromes of the respiratory tract such as bronchitis and asthma [9]. Because of its flavor, PCR, which is an herb of homology of medicine and food, has been extensively used as a condiment in many countries [10].

CRC mainly contains several compounds such as flavonoids, volatile oil and alkaloids [11–13]. Among these, flavonoids are considered to be responsible for the pharmacological activities of CRC. Research investigating the natural products has shown that flavonoids have wide-ranging biological activities, including inhibiting cell proliferation in cell cultures, inducing apoptosis, changing the activity of certain intracellular enzymes and functioning as antioxidants [14,15].

However, studies of CRC mainly focus on the absorption, distribution, metabolism and excretion of a few isolated bioactive compounds, such as hesperidin [16,17], nobiletin [18–20] and

Abbreviations: HPLC-PDA-IT-MS^n, high-performance liquid chromatography-photodiode array detector-ion trap mass spectrometry; CRCD, *Citrus reticulate 'Chachi'* decoction; HMF, 3,5,6,7,8,3′,4′-heptamethoxyflavone; TCM, traditional Chinese medicine; CID, collision-induced dissociation. TICs total ion current chromatograms; EICs, extracted ion chromatograms.

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tangeretin [20–23]. They can be absorbed into plasma after administration of monomer compounds, distributed to tissue, then metabolized and excreted from the organism. As we all know, TCM is a very complicate mixture of a multitude of chemical constituents. One or a few bioactive compounds used for in vivo studies cannot reflect its overall efficacy. Meanwhile, not all the compounds existed in the TCM or their prescriptions could be adsorbed into the organism, but only those being absorbed into blood can represent certain bioactivity. Therefore, it is very important to screen and analyze the bioactive components in TCM and pharmacology, not only for the quality control of crude drugs but also for elucidating the therapeutic principle of TCM. Qiao *et al.* [24] indicated that metabolic profiling can provide important evidences to clarify the mechanism of herb medicines.

Recently, liquid chromatography coupled with mass spectrometry (LC/MS) has demonstrated its great advantages of efficient chromatographic separation and sensitivity and specificity for qualitative analysis of compounds in TCM extracts and those metabolites via biotransformation [25–27]. In addition, some MS^n techniques such as IT-MS^n, TOF-MS (Q-TOF) and Q-Orbitrap-MS have made it possible to acquire rich structural information on analytes of interest. Nowadays, HPLC fingerprint and quantitative analysis of multi-components were often used for the quality research of CRC [13,28–31]. Only a few papers have reported the qualitative analysis of components in PCR by using LC-MS^n [32,33]. Ding *et al.* [32] reported the application of LC-HRMS and LC-MS^n for the identification of PCR and only nine components were definitely identified. And Zheng and co-workers [30] showed that 41 compounds in PCR was scanned by LC-MS/MS which was performed by a triple-Quadrupole instrument and 25 compounds of these were clearly identified. Otherwise, the reproducibility of triple-Quadrupole-MS analyzer for qualitative analysis is much worse than IT-MS^n, TOF-MS, etc.

In our study, a new method which combined HPLC-PAD-IT-MS^n and HPLC-Quadrupole-Orbitrap-MS with confirmation of the reference standards was used for identification of the bioactive components in CRCD. First, HPLC-PAD-IT-MS^n was selected to analyze chemical constituents of CRCD and SD rat plasma pretreated samples collected after oral administration of CRCD. Then, Q-Orbitrap-MS, a new technique from a Benchtop Quadrupole Orbitrap Mass Analyzer, which has much higher sensitivity and resolution than IT/MS^n and TOF/MS, was applied to further confirm the information of MS data of chemical constituents and those metabolites of CRCD that was acquired by IT/MS^n. As a result, 27 bioactive components in CRCD were identified, and the chemical fingerprint of CRCD was established. Meanwhile, a total of 43 compounds in SD rat plasma pretreated samples were identified or tentatively assigned, including 9 prototypes and 34 metabolites.

2. Experimental

2.1. Chemicals and reagents

Dried peels of *C. reticulata 'Chachi'*, Guangchenpi, were bought from local TCM Company in Guangdong province of China. The species was verified by Prof. Shuyuan Li (Department of Chinese Medicine of Guangdong Pharmaceutical University). The samples were ground into powder through a 50-mesh sieve. The powders were dried at 60 °C until constant weight and were well blended before use.

HPLC-grade acetonitrile and methanol from Merck (Darmstadt, Germany) were used. HPLC-grade formic acid was purchased from ROE Scientific Inc. (Newcastle, USA). Water for HPLC analysis was purified by Milli-Q academic water purification system (Millipore, MA, USA). Heparin sodium was from Shanghai No. 1 Biochemical and Pharmaceutical Co. Ltd and dissolved in physiological saline at the concentration of 50 mg/mL. It was used to rinse the test tubes prior to blood collection for plasma.

The reference standards of several compounds including hesperidin, hesperetin, nobiletin and tangeretin were all purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). And vicenin-2 was isolated in our laboratory from the peels of CRC, purity of which was over 95%.

2.2. Preparation of C. reticulate 'Chachi' decoction (CRCD) and standard solution

Two-hundred and fifty grams of dried crude powder of *C. reticulate 'Chachi'* was immersed in 2.5 L of 50% methanol overnight. The mixture was extracted twice by refluxing extraction for 2 h at 80°C, then filtered and collected the combined filtrates. After condensing the aqueous extracts into approximately 250 ml, the solution was passed through a 500 g macroporous resin column which had been washed with 2 L of 95% ethanol and then equilibrated with 5 L of water. The column was serially eluted with 2.5 L of water and 5 L of 95% ethanol. The 95% ethanol fraction was evaporated to dryness under reduced pressure at 60 °C. The dried residue was dissolved in water to obtain an oral solution of CRCD with a concentration of 1 g herb/mL decoction.

Hesperidin, hesperetin, nobiletin, tangeretin and vicenin-2 standard stock solution were prepared by dissolving approximately 10 mg each of accurately weighted pure compound in 10 mL of methanol, respectively.

2.3. Animal and biological sample collection

Sprague–Dawley rats were provided by Shanghai SLAC Laboratory Animal Co. Ltd. The animals were kept in an environmentally controlled breeding room for 7 days before starting the experiment and then fasted with free access to water *ad libitum* overnight before the test. All the animals used in this study were in compliance with institutional animal care guidelines, and the animal use and care protocols were approved by the Local Institutional Committee of the Second Military Medical University.

In order to research the dynamic changes of CRCD *in vivo* (mainly in blood), six healthy and mature Sprague–Dawley rats (male and female in half, 220 ± 10 g body weight) were selected to be orally administered at a single dose of 1.5 mL CRCD/kg body weight (equivalent to 1.5 g of crude drug/kg, according to Chinese Pharmacopoeia [2]). According to the reports, the peak time of several bioactive components of CRCD in rat plasma, such as hesperidin, nobiletin and tangeretin [16,18,23], was approximately 1–4 h. Therefore, a volume of 1.2 mL blood sample was collected before administration and after 1, 2, 4 and 8 h of oral administration ($n = 6$). Otherwise, 1 mL of water was fed to the rats every 1.5 h during the experiment. All blood samples were then centrifuged at 4000 rpm for 10 min at 4 °C, and the supernatants (i.e., the plasma) were stored at −80 °C until additional extraction and analysis.

In our study, biological samples were pretreated with solid-phase extraction (SPE) before HPLC analysis in order to reduce the matrix interference and/or concentrate the analytes. Before use, supelclean™ LC-18 SPE columns (1 mL/100 mg volume, Supelco, USA) were conditioned and equilibrated with 1 mL of methanol and 2 mL of deionized water, respectively.

The plasma samples (500 μL) were diluted with equal volume of water and then loaded onto the preconditioned SPE columns. After washed off with 2 mL of deionized water and 5% methanol, the SPE columns were eluted using 1 mL of methanol. The eluant was then evaporated to dryness under reduced pressure at 35 °C. The residues were dissolved in 50 μL of methanol, then fully vortexed