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Metabolite profiling analysis of FR429, an ellagitannin purified from *Polygonum capitatum*, in rat and human liver microsomes, cytosol and rat primary hepatocytes *in vitro*

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ABSTRACT

FR429, an ellagitannin (a type of polyphenol), is isolated and purified from *Polygonum capitatum* Buch.-Ham.ex D. Don which is the original herbal medicine of the "Re-Lin-Qing" formula used clinically to treat urinary tract infection in China. FR429 has been investigated for its antitumor potential in tumor-bearing nude mice *in vivo*, but its *in vitro* anti-tumor effect in hepatoma cell lines was low. Thus, it was of our interest to investigate its metabolism pathways for supporting its *in vivo* antitumor potential. The metabolic profiles of FR429 were studied *in vitro* by liquid chromatography coupled to ion trap time-of-flight mass spectrometry. Total eight metabolites were identified in rat and human liver microsomes, cytosol, and rat primary hepatocytes *in vitro*. Ellagic acid, a reported anti-angiogenic agent, was one of the main metabolites in these biological matrices. Methylated metabolites catalyzed by catechol-O-methyl transferase (COMT) were observed mainly in the *in vitro* incubation with rat liver cytosol, which was verified by using a COMT specific inhibitor entacapone and supported by molecular docking analysis. Methylated and sulfated metabolites were also found in rat primary hepatocytes in a time-dependent manner. In conclusion, the *in vitro* metabolism pathways of FR429 were hydrolysis, methylation and sulfation. The anti-tumor effects of its major metabolites should be further studied.

1. Introduction

Many medicinal plants, such as *Punicagranatum*, *Geranium thunbergii*, and *Cornusofficinali*, are rich in phenolic compounds known as ellagitannins (ETs). ETs are a family of bioactive polyphenols with large molecular weight, high polarity, and a core of glucose esterified with hexahydroxydiphenic acid. The antioxidant, antitumor, antiviral, antimicrobial, and immune-modulatory effects of ETs have been demonstrated [1,2]. Consumption of foods containing ETs, such as raspberries, pomegranates, and red wine, is recommended for the prevention of cardiovascular diseases and cancer due to their potent antioxidant properties. However, ETs are rarely detected after normal consumption of ET-rich foods due to their low bioavailability [3,4]. Whether ETs can be metabolized in liver has not been reported, and its possible metabolic pathways have not been elucidated completely.

ETs can be metabolized *in vitro* by gut bacteria into urolithins and their derivatives [5–7], and urolithins and their glucuronides have been observed in the plasma and urine of rats and human [8,4]. Under physiological conditions, ETs can also undergo hydrolysis to form ellagic acid (EA), which is an intermediate metabolite of ETs with low bioavailability [9]. EA can be further metabolized into urolithins by colonic microflora upon oral administration [10]. The metabolites of EA, including urolithins, their glucuronide conjugates and glucuronide conjugates of methylated EA, have been detected in rat plasma, urine, and bile [4,10]. EA and its metabolites, specifically including urolithins and dimethyl EA,

Abbreviations: COMT, catechol-O-methyl transferase; ET, ellagitannin; EA, ellagic acid; GA, gallic acid; LC/MS^n-IT-TOF, liquid chromatography-ion trap-time of flight mass spectrometry; SAM, S-adenosyl-L-methionine; MS^n, multi-stage mass spectrum.

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have been shown to exhibit potent anti-cancer, antimicrobial, and antioxidant activities *in vitro* and *in vivo* [7,11–13].

Polygonum capitatum Buch.-Ham.ex D. Don, a traditional Miao-nationality herbal medicine, is widely used to treat various urologic disorders including pyelonephritis and urinary tract infection. Its preparation (namely as Re-Lin-Qing granules) is widely used in clinics in China [14]. Previous studies have shown that ethanolic extracts of *P. capitatum* possess antibacterial, anti-inflammatory, and antioxidant activities [15,16]. FR429, a typical ET, is the most abundant component in ethanolic extracts of *P. capitatum* [17]. Like other ETs, FR429 can be biotransformed by intestinal microflora *in vitro*, generating metabolites such as gallic acid (GA), urolithins, and hydrolyzed products of FR429 [18].

Our previous studies have indicated that FR429 at very high concentrations (IC_{50} > 100 μM) induced apoptosis in several hepatoma cell lines *in vitro*, whereas it dramatically inhibited tumor growth in hepatoma-xenografted mice after intraperitoneal administration (10 mg/kg) for 2 weeks [19]. We are interested in studying the metabolic profiles of FR429 to determine whether the anti-cancer effects observed in xenografted mice are derived from its metabolites. In the current study, we used LC/MS^n-IT-TOF to analyze the metabolic profiles of FR429 in rat and human liver microsomes, cytosol and rat primary hepatocytes *in vitro*. In addition, since catechol polyphenols can undergo methylation by catechol-O-methyl transferase (COMT) [20], we hypothesized that FR429, as a type of polyphenol compound, should be methylated by COMT. Thus, the involvement of COMT in the metabolism of FR429 was also investigated via incubation with liver cytosol *in vitro* and molecular docking analysis *in silico*.

2. Materials and methods

2.1. Chemicals and reagents

The authentic standards of ellagic acid (EA), gallic acid (GA) and emodin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), antibiotics, antimycotic, and trypsin were purchased from Gibco (Grand Island, NY, USA). β-nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PD), dithiothreitol, and S-adenosyl-L-methionine (SAM) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Entacapone was purchased from Kangbaotai Fine-Chemicals Co., LTD (Hubei, China). HPLC grade acetonitrile and formic acid were obtained from Labscan Analytical Science (Bangkok, Thailand), and ethyl acetate was from Fisher Chemicals (Leicester, UK). Distilled and deionized water was prepared using a Milli-Q purification system. The pooled human liver cytosol (Lot number QROX; from 7 donors) and microsomes (Lot number KQB; from 50 donors) were supplied by Research Institute for Liver Diseases Co., Ltd (Shanghai, China) from Celsis *In Vitro* Technologies Inc. (MD, USA), and stored at −80 °C before use. All other un-specified chemicals were purchased from Sigma Chemical Co.

2.2. Purification of FR429

FR429 was isolated according to the previous report with slight modification [17]. Briefly, powders of dried plant (20 g) were extracted with 70% ethanol (500 ml) for 12 h by cold maceration, and then extracted by ultrasonic for 1 h. After filtration and removal of the solvent by evaporation under vacuum, the residue was re-suspended in 100 ml distilled water and then extracted five times by equal volume of ethyl acetate. The combined ethyl acetate solution was concentrated under reduced pressure at 45 °C to obtain dry residue. The residue redissolved in methanol was subjected to elution with methanol using a Sephadex LH20 column before the brown fraction (FR429) was obtained. This fraction was evaporated for dry product with purity of 98%, as determined by HPLC-UV. MS and NMR were also used for identification and determination of FR429 as previous study [18].

2.3. LC/MS^n-IT-TOF analysis

An HPLC coupled to an ion trap time-of-flight mass spectrometer (LC/MS^n-IT-TOF, Shimadzu Cooperation, Tokyo, Japan) was used for the identification of FR429 and its metabolites. FR429 and its metabolites were separated using an Alltima C18 column (150 mm × 4.6 mm, 5 μm). The mobile phase was composed of acetonitrile (A) and 0.2% formic acid (B) (v/v) at a flow rate of 0.8 ml/min with a gradient elution: 0–10 min, 10% A; 30 min, 30% A; 40 min, 65% A; 45 min, 85% A; and 55 min, 85% A. The detection wavelengths were set at 254 nm and 280 nm. The column temperature was set at 25 °C. For LC/MS^n-IT-TOF analysis, an electrospray ionization (ESI) resource with a negative mode was used. Nitrogen was used as the nebulizing gas, and helium was used for fragmentation of the MS^n analyte. Other parameters were set as follows: curved desolvation line (CDL) temperature, 200 °C; heat block temperature, 200 °C; detector voltage, 1.70 kV; nebulizing gas, 1.5 L/min; and drying gas pressure, 110 kPa. The energy of collision-induced dissociation (CID) was set at 50%. Mass spectra were acquired for MS^1 in the range of *m*/*z* 100–1000. The MS^n data were collected in an automatic mode. Three MS^n stages were set, and the spectra were acquired in the range of *m*/*z* 100–800 for MS^2, 100–700 for MS^3, and 100–500 for MS^4, respectively.

2.4. Preparation of rat liver microsomes and cytosol

Preparation of rat liver microsomes and cytosol was performed as described previously [21]. In brief, after general anesthesia, rats were sacrificed. The liver was excised, rinsed with ice-cold normal saline, weighed, and homogenized in 0.1 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged at 10,000*g* at 4 °C for 30 min, and the supernatant was then centrifuged at 105,000*g* at 4 °C for 60 min. Finally, the supernatant was used as the cytosol fraction, and the pellet was reconstituted in 0.1 mM phosphate buffer (pH 7.4) for microsomes. Samples were then kept at −80 °C until use. Protein concentrations of liver microsomes and cytosol were determined by using a BCA protein assay kit (Thermo, IL, USA).

2.5. Incubation of FR429 in human and rat liver microsomes and cytosol

In order to obtain the most abundant metabolites, the incubation conditions, including protein contents, FR429 concentrations and time slots, have been optimized. The optimized incubation conditions used for human and rat microsomes incubation were as follows: 100 μM FR429, 1 mM NADP, 5 mM G-6-P, 2 units/ml G-6-PD, and 5 mM magnesium chloride. The final incubation volume (250 μl) was achieved with Tris/KCl buffer (pH 7.4). After pre-incubation for 5 min at 37 °C, microsomes (6 mg/ml as final concentration) were added to initiate the 2-h reaction at 37 °C, which was then terminated with ice-cold acetonitrile (250 μl). After centrifuged at 10,000*g* for 10 min, the supernatant was dried using nitrogen gas. The residues were dissolved in an 80-μl mobile phase, and a 30-μl aliquot was analyzed by LC/MS^n-IT-TOF.

The optimized incubation conditions (200-μl reaction volume) used for human and rat cytosol incubation were as follows: