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Chemical and biological assessment of Jujube (*Ziziphus jujuba*)-containing herbal decoctions: Induction of erythropoietin expression in cultures

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ABSTRACT

Jujubae Fructus, known as jujube or Chinese date, is the fruit of *Ziziphus jujuba* (Mill.), which not only serves as daily food, but acts as tonic medicine and health supplement for blood nourishment and sedation. According to Chinese medicine, jujube is commonly included in herbal mixtures, as to prolong, enhance and harmonize the efficiency of herbal decoction, as well as to minimize the toxicity. Here, we aim to compare the chemical and pharmacological properties of three commonly used jujube-containing decoctions, including Guizhi Tang (GZT), Neibu Dangguijianzhong Tang (NDT) and Zao Tang (ZOT). These decoctions share common herbs, i.e. Glycyrrhizae Radix et Rhizoma Praeparata cum Melle, Zingiberis Rhizoma Recens and Jujube, and they have the same proposed hematopoietic functions. The amount of twelve marker biomolecules deriving from different herbs in the decoctions were determined by LC–MS, and which served as parameters for chemical standardization. In general, three decoctions showed common chemical profiles but with variations in solubilities of known active ingredients. The chemical standardized decoctions were tested in cultured Hep3B cells. The herbal treatment stimulated the amount of mRNA and protein expressions of erythropoietin (EPO) via the activation of hypoxia response elements: the three herbal decoctions showed different activation. The results therefore demonstrated the hematopoietic function of decoctions and explained the enhancement of jujube function within a herbal mixture.

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1. Introduction

JujubaFructus (JF), also known as jujube or Chinese date, is the fruit of *Ziziphus jujuba* (Mill.), and which is considered as one of five valuable fruits in China. Jujube serves as daily food, as well as being prescribed as a tonic medicine for blood nourishment and sedative effect. In recent studies, jujube was reported to have various functions, including anti-oxidation [1,2], neuro-protection [3,4], improving cardiovascular system [5], anti-microbial [6], anti-inflammatory effect [7], anti-ulcer [8] and anti-cancer [9]. Several lines of evidence support the blood nourishing function of jujube [5,10]. According to Chinese medicine, blood nourishment includes blood formation (hematopoiesis), oxygenation and controlling factors for formation of red blood cells. Indeed, many herbal decoctions were demonstrated to facilitate blood formation, and different cell models were established to investigate this function [11]. Erythropoietin (EPO), a glycoprotein involving in erythropoiesis [12], is produced by embryonic liver and adult kidney cells. Under hypoxia, when the oxygen sensors in liver and kidney detect the low oxygen level, this will trigger the production of EPO, as to increase amount of red blood cells and hence to restore oxygen level [13,14]. This response is regulated by a hypoxia response element (HRE) signaling pathway. HRE is an enhancer sequence located on promoter region of EPO gene, and the activation of HRE initiates expression of EPO [15–17].

Jujube is frequently being included in a herbal formulated decoction, which is aiming to enhance the medicinal values, to facilitate

Abbreviations: ASR, Anelicae Sinensis Radix; CC, Cinnamomi Cortex; CR, Cinnamomi Ramulus; EPO, erythropoietin; GRRPM, Glycyrrhizae Radix et Rhizoma Praeparata cum Melle; HIF, hypoxia induced factor; GZT, Guizhi Tang; HRE, hypoxia response element; IS, internal standard; JF, JujubaFructus; NDT, Neibu Dangguijianzhong Tang; PAR, Paeoniae Alba Radix; ZOT, Zao Tang; ZRR, Zingiberis Rhizoma Recens.

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the absorption and/or to reduce the toxicity of individual herbs. According to Chinese medicinal theory, a herbal decoction normally comprises of four elements, including "*Jun*" (prime), "*Chen*" (minister), "*Zuo*" (assistant) and "*Shi*" (servant): the four elements are acting together to harmonize the therapeutic functions [18–20]. Jujube is widely included in numerous herbal decoctions serving as assistant or servant herb. Among these jujube-containing herbal mixtures, many of them are popularly used today for blood nourishing functions.

Guizhi Tang (GZT), composed of Cinnamomi Ramulus (CR; Guizhi), Paeoniae Alba Radix (PAR; Baishao), Glycyrrhizae Radix et Rhizoma Praeparata cum Melle (GRRPM; Zhigancao), Zingiberis Rhizoma Recens (ZRR; Shengjiang) and JujubaeFructus (JF; Zao), was prescribed in Shang Han Lun by Zhang Zhongjing in Han Dynasty (∼200 AD). Neibu Dangguijianzhong Tang (NDT), composed of Angelicae Sinensis Radix (ASR; Danggui), Cinnamomi Cortex (CC; Rougui), Paeoniae Alba Radix (PAR; Baishao), Glycyrrhizae Radix et Rhizoma Praeparata cum Melle (GRRPM; Zhigancao), Zingiberis Rhizoma Recens (ZRR; Shengjiang) and JujubaeFructus (JF; Zao), was prescribed in Bei ji Qian jin Yao Fang by Sun Simiao in Tang Dynasty (652 AD). Zao Tang (ZOT), composed of Glycyrrhizae Radix et Rhizoma Praeparata cum Melle (GRRPM; Zhigancao), Zingiberis Rhizoma Recens (ZRR; Shengjiang) and JujubaeFructus (JF; jujube), was prescribed in Formulae of the Pharmacy Service for Great Peace and for the Benefit of the People by Official Bureau of Physicans (Taiyi ju) in Sung Dynasty (1078–1085 AD). Although the three decoctions are prescribed in different dynasties, they are considered as "Jia Jian Fang" of having modification by adding herbs on top of a basic formula, and thus the three jujube-containing decoctions are sharing herbal compatibility with similar functions.

Here, we hypothesize that different decoctions should have a variation in hematopoietic function, i.e. stimulating EPO expression. Chemical parameters were established here to standardize the herbal decoctions, and subsequently they were tested functionally in cultured Hep3B liver cells to reveal mRNA and protein expressions of EPO, as well as the transcriptional activity of HRE.

2. Materials and methods

2.1. Materials

The fruits of *Z. jujuba* cv. Jinsixiaozao (JF) from Hebei of China were collected in 2012. The dried stem bark and dried young branch of *Cinnamomum cassia* Presl (CC and CR) were collected from Guangxi China. The roots of *Angelica sinensis* (Oliv) Diels. (ASR), the processed root of *Paeonia lactiflora* Pall. (PAR), the root and rhizome of *Glycyrrhiza uralensis* Fisch. or *Glycyrrhiza inflata* Bat. under the method for stir-baking with honey (GRRPM), the rhizome of *Zingiber officinale* Roscoe (ZRR), were collected from Gansu, Anhui, Inner Mongolia and Guangdong of China. The raw materials were purchased from herbal markets. No specific permissions were required for collection of raw materials, and the location was also not privately-owned or protected. The plant materials were authenticated by Dr. Tina Dong based on their morphological characteristics and deposited in the Center for Chinese Medicine, The Hong Kong University of Science and Technology. The dried jujube was chopped into half, and ginger was chopped into slices according to ancient preparation method. cAMP (1) and cGMP (2) were purchased from Sigma–Aldrich (St. Louis, MO). Rutin (4) and astilbin (IS) were purchased from Tauto Biotech (Shanghai, China). Formononetin (5) and calycosin (6) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Paeoniflorin (3), cinnamic acid (7), glycyrrhizic acid (8), *Z*-ligustilide (10) and ferulic acid (12) was purchased from The Testing Laboratory for Chinese Medicine (Hong Kong, China). 6-Gingerol (9) was purchased from Sichuan Weikeqi Biological Technology Co., Ltd. (Sichuan, China). Liquiritin (11) was purchased from Shanghai R&D Center for Standardization of Chinese Medicine (Shanghai, China). The purity of all marker biomolecules were determined to be over 98% by normalization of peak areas, as revealed by HPLC–DAD. MS-grade acetonitrile and water were purchased from Merck (Darmstadt, Germany). Ultra-pure water was prepared using a Mili-Q purification system (Molsheim, France) HPLC-grade methanol was purchased from Merck. Other reagents used here were of analytical grade.

2.2. Preparation of herbal extracts

Total herbs (25 g) were weighted according to the formulation and were boiled together under moderate heating in 20 vol of water for 1 h, twice. The combined extracts were dried into powder under vacuum by lyophilizer at −80 °C. Before the assessments, the powder was re-dissolved with water to a concentration of 10 mg/mL and 100 mg/mL, as stock solutions for chemical and biological assessments, respectively. For chemical assessment under LC–DAD–MS/MS and HPLC system, the extracts were filtered through a 0.45-μm membrane filter before the analysis. For biological assessments, the extracts were filtered through a 0.22-μm membrane filter before the cell treatment.

2.3. Chromatographic conditions and MS/MS analysis

Agilent RRLC 1200 series system (Waldron, Germany) equipped with a degasser, a binary pump, an auto-sampler, a diode array detector (DAD) and a thermo-stated column compartment was adopted for establishment of fingerprint for herbal extracts. The mobile phase consisted of 0.03% phosphoric acid in water (A) and acetonitrile (B), respectively. The extracts were separated on an Agilent ZORBAX SB-Aq (4.6 × 250 mm, 5 μm) C18 column, and a wavelength of 210 nm were adopted for measurement. For MS analysis, the extracts were separated on an Agilent Eclipse Plus C_{18} RRHD (2.1 × 50 mm column, 1.8 μm) column by Agilent RRLC 1200. The mobile phases were composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), respectively. A elution gradient was set up as follows: 0–2 min isocratic gradient 99% (A) with flow rate of 0.3 mL/min; 2–4 min, linear gradient 99 → 90% with flow rate of 0.3 mL/min; 4–8.75 min, linear gradient 90% → 80% (A) with flow rate of 0.3 mL/min; 8.75–10 min, isocratic gradient 80% (A) with flow rate of 0.3 mL/min; 10–17 min, linear gradient 80% → 70% (A) with flow rate of 0.3 mL/min; 17–22 min, linear gradient 70% → 65% (A) with flow rate of 0.3 mL/min; 22–30.75 min, linear gradient 65% → 30% (A) with flow rate of 0.3 mL/min; 30.75–38 min, isocratic gradient 30% (A) with flow rate changing from 0.3 to 0.2 mL/min. A pre-equilibration period of 3 min was used between each run. The column temperature was set to 25 °C. The injection volume was 10 μL. A wavelength of 210 nm was employed for analysis.

Agilent QQQ-MS/MS system equipped with an ESI ion source was operated in both positive and negative ion modes. The drying gas temperature was 325 °C; drying gas flow: 10 L/min; nebulizer pressure: 35 psig; capillary voltage: 4000 V; delta electro multiplier voltage: 400 V. One to two suitable transition pairs was chosen for acquisition in multiple reactions monitoring (MRM) mode for selected markers and internal standard astilbin. The fragmentor voltage and collision energy values were optimized to obtain the highest abundance. Agilent MassHunter Workstation software version B.06.00 was used for data acquisition, processing and analysis.

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