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Identification of bioactive ingredients with immuno-enhancement and anti-oxidative effects from Fufang-Ejiao-Syrup by LC–MS^n combined with bioassays

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ABSTRACT

Fufang Ejiao Syrup (FES) is a widely used immune-boosting Traditional Chinese Medicine (TCM) in Eastern Asian countries. This study attempts to investigate the bioactive compounds in FES. First, FES extract was separated into fractions to facilitate the investigation and 72 compounds were identified using LC–MS^n. Subsequently, Immune-enhancement effects of FES and its components were investigated on bone marrow cells and neuroprotective effects against H_2O_2 induced oxidative damage were evaluated in SH-SY5Y neuroblastoma cells and bEnd.3. Our results indicated that fraction 3, 5, 6 and 8 showed significant improvements on immune function, while several fractions had cytoprotective effects against H_2O_2-induced oxidative injury. Jionoside A1 isolated from *Radix Rehmanniae Praeparata* displayed dose dependent immune-enhancement activity. 20(*R*)-ginsenoside Rg_3 could protect bEnd.3 against oxidative damage. Furthermore, echinacoside, jionoside A1, vitexin-2-*O*-rhamnoside, acteoside and isoacteoside possessed moderate protective activities on H_2O_2-treated SH-SY5Y cells. In conclusion, our study provided both chemical and biological evidences to support clinical application of FES.

1. Introduction

Nowadays, people suffering from sub-health are prone to dizzy, fatigue and insomnia. For these multi-symptoms, Traditional Chinese Medicine (TCM) becomes a preferred choice to meet the requirement of daily life and improve physiological and psychological well-being of patient [1,2]. In Eastern Asian countries including China, Japan and Korea, Fufang Ejiao Syrup (FES) has been reported with multiple beneficial functions on improving the sub-healthy state of human body, such as alleviating fatigue [3], ameliorating anemia [4] and boosting immunity [5]. The increasing reputation and mounting sales of FES have stimulated the generation of generic products. On the other hand, lack of essential mechanistic study seriously hindered the implementation of quality assurance and further application of FES. Therefore it is necessary to investigate chemical constituents of FES and identify ingredients with beneficial properties.

To date, systematic studies of the chemical constituents and its applications associated activities of FES was rather limited. FES was consisted of extracts of five traditional Chinese medicine, *i.e., Radix Ginseng Rubra, Radix Rehmanniae Preparata, Codonopsis pilosula, Crataegus pinnatifida Bge, and Colla corii asini.* The peptides of *Colla corii asini* could promote hematopoiesis by activating immature granulocyte and erythroid cells [6]. *Radix Ginseng Rubra* and *Codonopsis pilosula* had anti-oxidative [7] and immune-modulatory activity [8]. *Radix Ginseng Rubra* also demonstrated anti-bacterial, anti-obesity and anti-diabetic activities [9]. A number of studies have validated the blood enriching effect and anti-fatigue activity of *Radix Rehmanniae Preparata* polysaccharides [10]. It is also reported that *Crataegus pinnatifida Bge* extract had antioxidant activity [11] and neuroprotective effect [12]. These previous works have provided valuable information about bioactivities of FES, which would facilitate further systematic investigations on this TCM preparation.

In this study, we applied high performance liquid chromatography–mass spectrometry (HPLC–MS) together with high resolution-mass spectrometry (HR-MS) technologies to characterize the phytochemical constituents of FES. Fractions of FES were prepared and several ingredients were screened for pharmacological effects based on *in vitro* cellular models.

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2. Material and methods

2.1. Chemicals and herb materials

The aqueous extract of FES was supplied with Donge Ejiao Co., Ltd, which has already been excluded of peptide and proteins constituents. The MPLC system was constructed by a Büchi 688 chromatography pump and a chromatography column prepared with RP-C_{18} silica gel (YMC GEL ODS-A-HG S-50 μm 12 nm, YMC Co., Ltd., Tokyo, Japan).

Analytical-reagent grade methanol was purchased from Yonghua Chemical Technology Co., Ltd. (Changshu, Jiangsu, China). Deionized water was prepared by Milli-Q system (Millipore, Bedford, MA, USA). HPLC-grade acetonitrile (Merck KGaA, Darmstadt, Germany) and formic acid (TEDIA, Fairfield, OH, USA) were utilized for the HPLC analysis. All other chemicals and solvents were analytical reagent grade. Wogonoside, vitexin-2-*O*-rhamnoside, hyperoside, echinacoside, acteoside, isoacteoside, chlorogenic acid and baicalin were purchased from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China). *R*-notoginsenoside R_2 was purchased from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). Ginsenoside Rf, 20(*S*)-ginsenoside Rg_2, 20(*R*)-ginsenoside Rg_2, 20(*S*)-ginsenoside Rg_3, 20(*R*)-ginsenoside Rg_3, ginsenoside Ro, ginsenoside Rb_1 and ginsenoside Re were purchased from Jilin University (Jilin, China). Lobetyolin was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Jionoside A1, jionoside B1, jionoside B2, martynoside and purpureaside C [13] were isolated from *Radix Rehmanniae Preparata* by Pro. Jizhong Yan of Zhejiang University of Technology.

SH-SY5Y cells was purchased from Shanghai Cell Bank of Chinese Academy of Sciences. Microvascular endothelial cell line (bEnd.3) cells were obtained from American Type Culture Collection (ATCC, USA). DMEM with 1 g/L glucose (Hyclone Laboratories, UT, USA). Fetal Bovine Serum (FBS, Gibco, USA). 1% penicillin/streptomycin (Gibco, USA). DMSO (Sigma, USA). MTT solution (Sigma, USA). CCK-8 (Beyotime, Jiangsu, China). CO_2 incubator (Thermo, USA). Microplate reader (Tecan F200, USA). Leica DMI6000B inverted microscope with Leica digital camera (Germany).

2.2. Sample preparation

The aqueous extract of FES was evaporated by a rotary evaporator at 40 °C with a high vacuum lyophilizer. The crude black solid mass (10 g) was then fractionated using the RP-C_{18} silica gel column (the eluted volume was optimized for obtaining good reproducibility, and described in Supplementary material Table S1), and the fractions were concentrated under reduced pressure for identification and pharmacological activity evaluation. FES and fraction solutions were preprocessed with centrifugation and filtration for HPLC–MS^n analysis.

2.3. Methods for chemical identification

Chromatographic separation was performed on an Agilent 1100 Series (Agilent, Germany) HPLC system equipped with a binary pump, an auto sampler, and a thermostatically controlled column apartment. Chromatographic separation was carried out at 25 °C on a ZORBAX SB-C_{18} column (4.6 × 250 mm, 5.0 μm). The mobile phases were 0.02% formic acid water (A) and 0.02% formic acid acetonitrile (B) using a gradient elution of 5–15% B at 0–10 min, 15% B at 10–20 min, 15–20% B at 20–50 min, 20–40% B at 50–110 min, 40–85% B at 110–125 min, 85–100% B at 125–126 min and 100% B at 126–135 min. The flow rate was kept at 0.500 mL/min. The injection volume was 20 μL.

For HPLC–MS^n analysis, a Finnigan LCQ Deca XP^{plus} ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) was connected to the Agilent 1100HPLC instrument via an ESI interface. The parameters of the ESI source were as follows: collision gas, ultra-high purity helium (He); ion spray voltage, −4.0 KV; sheath gas (N_2), 60 arbitrary units; auxiliary gas (N_2), 20 arbitrary units; capillary temperature, 350 °C; capillary voltage, −19 V; tube lens offset voltage, −25 V. The collision energy for collision-induced dissociation (CID) was 20–50%. The LC–MS^n data was analyzed by Xcalibur 2.1, Qual Browser (Thermo Fisher Scientific Inc., San Jose, CA, USA).

HR-MS data was obtain from a Waters ACQUITY UPLC (Waters Crop., Milford, MA, USA) equipped with an AB Triple TOF 5600^{plus} System (AB SCIEX, Framingham, MA, USA). 50 psi gas 1 (N_2) and gas 2 (N_2), 30 psi curtain gas, −4.5 kV ionization voltage and 550 °C source temperature were used in negative ion mode. The scan range was set at *m/z* 100–1500. Error tolerance was set to ±5 ppm. The injection volume was 7.5 μL. The liquid chromatography condition was same as described above. PeakView 1.2.0.3 workstation (AB SCIEX, Framingham, MS, USA) was applied to analyze the raw data.

2.4. Cell culture and bioactivity assays

2.4.1. Immune-enhancement effect on bone marrow cells

Specific pathogen free, 5- to 6-week old female BALB/C mice were purchased from Shanghai Laboratory Animal Company (SLAC, Shanghai, China) and maintained in the animal facility at Zhejiang University, China. The protocol obeyed the animal ethical standards and was approved by Animal Ethics Committee of Zhejiang University.

Mice were intraperitoneal injected with one dose 200 mg/kg of body weight of 5-fluorouracil (5-FU). Two days after injection, mice were sacrificed and suspensions of primary bone marrow cells were prepared and plated in 6-well microplates at a density of 3.5×10^5 cells per well in 2.5 mL of culture medium (DMEM complemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin) containing 50 μg/mL fractions [14] or 50 μM compounds [15] or left untreated (considered as control). Cells were incubated at 37 °C, 5% CO_2. Every week, half of the supernatants were removed and replaced by fresh culture medium with fractions or compounds or not. The cell growth was observed under inverted microscope weekly for four consecutive weeks. After 4 weeks' culture, the morphology of cells was observed and digitally photographed under an inverted microscope.

To evaluate the dose dependence of potential active compounds, the bone marrow stem cells of the original generation were seeded in 96-well microplates with 1000 cells/well and cultured in medium supplemented with different concentrations of potential compounds (0, 0.1, 1, 10, 25 and 50 μM, respectively) in a humidified incubator with 5% CO_2 at 37 °C. On day 3 or day 6, cell proliferation was assayed by measuring the colorimetric CCK8 on an ultra-microplate reader at 450 nm.

2.4.2. Antioxidant activity evaluation in H_2O_2 induced injury bEnd.3 model

bEnd.3 cells were grown in 90% DMEM and 10% FBS with penicillin/streptomycin (1%), at 37 °C in a humidified atmosphere containing 5% CO_2. bEnd.3 in exponentially growing stage were seeded into 96-microplates as 5×10^2 cells per well and were allowed to adhere for 24 h. Then, 10 μL of fractions (50 μg/mL) were added and co-incubated for 24 h. Cells were incubated with 0.5 mg/mL MTT for 4 h at 37 °C, and added 100 μL DMSO after MTT was discarded. The optical density (OD) was measured at 550 nm by the microplate reader. The mono compounds (50 μM) [16] were evaluated in the same way. Three wells were used for each sample.