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Chemical profiling of Huashi Baidu prescription, an effective anti-COVID-19 TCM formula, by UPLC-Q-TOF/MS

WEI Wen-LongΔ, WU Shi-FeiΔ, LI Hao-Jv, LI Zhen-Wei, QU Hua, YAO Chang-Liang, ZHANG Jian-Qing, LI Jia-Yuan, WU Wan-Ying, GUO De-An*

Shanghai Research Center for Modernization of Traditional Chinese Medicine, National Engineering Laboratory for TCM Standardization Technology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

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[ABSTRACT] Huashi Baidu prescription (HSBDF), recommended in the Guideline for the Diagnosis and Treatment of Novel Coronavirus (2019-nCoV) Pneumonia (On Trials, the Seventh Edition), was clinically used to treat severe coronavirus disease 2019 (COVID-19) with cough, blood-stained sputum, inhibited defecation, red tongue etc. symptoms. This study was aimed to elucidate and profile the knowledge on its chemical constituents and the potential anti-inflammatory effect in vitro. In the study, the chemical constituents in extract of HSBDF were characterized by UPLC-Q-TOF/MS in both negative and positive modes, and the pro-inflammatory cytokines were measured by enzyme-linked immunosorbent assays (ELISA) to determine the effects of HSBDF in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. The results showed that a total of 217 chemical constituents were tentatively characterized in HSBDF. Moreover, HSBDF could alleviate the expression levels of IL-6 and TNF-α in the cell models, indicating that the anti-viral effects of HSBDF might be associated with regulation of the inflammatory cytokines production in RAW264.7 cells. We hope that the results could be served as the basic data for further study of HSBDF on anti-COVID-19 effect.

[KEY WORDS] Huashi Baidu prescription; Corona virus disease 2019; Characterization of chemical constituents; ELISA

Introduction

Corona Virus Disease 2019 (COVID-19), a highly pathogenic Human coronaviruses (hCoVs), could cause viral pneumonia and pulmonary infection. COVID-19 possessed long latency, strong infectivity and difficult to cure features etc., which threaten the people’s health, economic development and social harmony [1]. By October 21, 2020, more than 40 000 000 people have been firmly diagnosed with COVID-19 around the world according to the statistical information of 1point3acres (https://coronavirus.1point3acres.com/), and there has been still no effective drugs to cure the devastating disease. According to the ancient Chinese documents and clinical experience, traditional Chinese medicine (TCM) had a good curative effect on viral pneumonia. National health and health commission (PRC) have updated and released in the Guideline for the Diagnosis and Treatment of Novel Coronavirus (2019-nCoV) Pneumonia (On Trials, the Seventh Edition), and the TCMs were recommended for prevention and treatment of COVID-19. In the Seventh Edition, Huashi Baidu Fang (HSBDF) consists of 14 herbal medicines (Ephedrae herba, Armeniacae semen amarum, Gypsum fibrosum, Glycyrrhizae radix et rhizoma, Agastache rugosus, Magnoliae officinalis cortex, Atractylodis rhizoma, Tsaoko fructus, Pinelliae rhizoma praeparatum, Poria, Rhei radix et rhizoma, Astragali radix, Descurainiae semen lepidii semen, Paeoniae radix rubra) were recommended and used to treat the critical patients with COVID-19 infection, and exhibited good therapeutic effect in clinical practice [2]. HSBDF was developed referred to multiple classical prescriptions (Ma Xing Shi Gan Decoction, Xuan Bai Cheng Qi Decoction, Dayuanyn, Huoxiangzhengqi San, Taoren Chengqi Decoction, Tingli Dazao Xiefei Decoction) for treatment of fever, cough, nausea, etc. Ma Xing Shi Gan Decoction and Xuan Bai Cheng Qi Decoction mainly used for facilitating lung and...
The expression levels of IL-6 and TNF-α in the cell supernatant were measured by ELISA. The data suggested that the anti-virus activity of HSBDF might be associated with an attenuated pro-inflammatory cytokine response.

**Experimental**

**Materials and reagents**

HPLC-grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). Formic acid (FA) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and deionized water (18.2 MΩ·cm) was prepared by a Millipore Alpha-Q water purification system (Bedford, USA). The herbal materials of ephedrae herba (voucher specimen number: EW20200601), atractylodis rhizoma (EW20200601), glycyrrhizae radix et rhizoma (GA20200601), magnoliae officinalis cortex (MO20200601), magnoliæ officinalis cortex (MO20200601), and paeoniae radix rubra (PRR20200601) were purchased from the National Institutes of Food and Drug Control (Beijing, China). Eight reference standards (ephephrine, pseudonephrine, liquiritin, glycyrrhizic acid, aloe-emodin, rhein, physcion, quercetin-3-O-β-D-glucosyl-7-O-β-D-gentiobioside) were purchased from Shanghai Nature Standard Technical Service Co., Ltd. (Shanghai, China). The Purity of all reference standards was more than 98%.

The extracted liquid of HSBDF for cell treatment was obtained as described above, which was finally adjusted to 10 μg μL⁻¹ with phosphate buffered saline (PBS) filtered through a 0.22 μm syringe filter, and stored at −20 °C until use. Lipopolysaccharides (LPS, from Escherichia coli serotype O55: B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO) were purchased from Weikeqi Biological Technology Co., Ltd. (Shanghai, China). All the herbal materials were authenticated by Professor GUO De-An following the method described in China Pharmacopeia (2015 edition), and the voucher specimens were deposited at the authors’ lab in Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China).

**Materials and reagents**

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   - Formic acid (FA) was purchased from Sigma-Aldrich (St. Louis, MO, USA).
   - Deionized water (18.2 MΩ·cm at 25 °C) was prepared by a Millipore Alpha-Q water purification system (Bedford, USA).
   - The herbal materials of ephedrae herba (voucher specimen number: EW20200601), atractylodis rhizoma (EW20200601), glycyrrhizae radix et rhizoma (GA20200601), magnoliæ officinalis cortex (MO20200601), and paeoniae radix rubra (PRR20200601) were purchased from the National Institutes of Food and Drug Control (Beijing, China).
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The ELISA kits for IFN-β, IL-6 and TNF-α were purchased from the Boster Bioengineering Institute (Shanghai, China).

Sample preparation

Sample preparation for characterization of chemical constituents in HSBDF: an amount of herbal materials was accurately weighed according to the prescription dose. The herbal materials (ephedrae herba, armeniacae semen amaram, gypsum fibrosum, glycyrhizae radix et rhizoma, magnoliae officinalis cortex, atractylodis rhizoma, tsaooko fructus, pinelliae rhizoma praeparatum, poria, astragali radix, descurainiae semen lepidii semen, paconiae radix rubra) were added into gallipot, and soaked for 10 minutes with ten times the amount of water. Then the herbal materials (agastache rugosus and rhei radix et rhizoma) were added when the water began to boil slightly. The prescription was decocted twice, and each time for 30 min. The extract was merged and dried by freeze drying. The 0.1 g HSBDF freeze dried sample was weighed and added 10 mL methanol–water (1 : 1, V/V) to prepare test solution, the supernatant was centrifuged at 14 000 r min⁻¹ before LC-MS analysis.

UPLC-Q-TOF/MS analysis conditions

Chromatographic separation of HSBDF was performed on a Waters ACQUITY I-Class UPLC® system (Waters Corporation, Milford, MA, USA) equipped with an ACQUITY UPLC® HSS T3 column (1.8 μm, 2.1 mm × 100 mm). The HSBDF was eluted by a binary mobile phase composed of acetonitrile (B) and 0.1% formic acid (V/V; A) following the gradient elution program: 0–20 min: 0–60% B; 20–22 min: 60%–90% B; 22–25 min: 90%–90% B. The flow rate was 0.3 mL·min⁻¹, the column temperature was set at 30 °C, and 5 μL of the test solution was injected for analysis.

High-resolution profile MS data were acquired on a Waters Xevo® G2-S QTOF mass spectrometer (Waters, Manchester, UK) connected to the UPLC system via a Zspray™ ESI source. The mass range of m/z 150–1500 was set for full-scan, and the collision energy ramp of 15–25 V and 35–45 V were set for low mass and high mass, respectively. Capillary voltages of 2 kV, cone voltage of 40 V, cone gas flow of 30 L h⁻¹, source temperature of 140 °C, and desolvation gas flow of 700 L h⁻¹ at 500 °C were utilized. A solution of leucine-enkephalin (1 μg mL⁻¹) was used as lock mass for data calibration.

Cell culture

Murine macrophage cell line (RAW264.7) was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. RAW264.7 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U·mL⁻¹ penicillin G and 100 μg·mL⁻¹ streptomycin) (Gibco). Cells were cultured under standard conditions (5% CO₂ in air in a humidified environment at 37 °C).

Cytotoxicity assay

RAW264.7 cells were placed in 96-well plates at a density of 4 × 10⁴ cells/mL for 24 h and then treated with different concentrations of HSBDF. Cell viability was measured by the MTT assay method according to the instructions. The absorbance was measured at 490 nm with a microplate reader (Thermo Scientific Varioskan LUX). The normal control (cells with no treatment) was set at 100%, and the treated samples were normalized to this value. The 50% cytotoxic concentration (CC₅₀) was calculated as the concentration of HSBDF causing the death of 50% of the cells.

Determination of inflammatory cytokines by enzyme-linked immunosorbent assay (ELISA)

The cells were seeded in 12-well plates (3 × 10⁵ cells/well) and were co-treated with HSBDF (20 and 100 μg·mL⁻¹) in the presence of LPS (1 μg·mL⁻¹) (except for the Blank group) for 4 or 12 h. After centrifugation, cell-free supernatants were collected for assaying TNF-α, IL-6 production. The secretion amount of TNF-α, IL-6 in the media were measured through ELISA kits (Boster, Shanghai, China) by following the standard protocol. The 450 nm absorbance was tested through a microplate reader (Thermo Scientific Varioskan LUX).

Data processing

MassLynx V4.1 software (Waters, Milford, USA) was employed in data acquisition and processing. One-way analysis of variance (ANOVA) and Student’s t-test were used to assess differences between the treatment groups. The P values less than 0.05 were considered as statistically significant. The CC₅₀ values were calculated by nonlinear regression in GraphPad Prism 5 (San Diego, CA, USA).

Results and Discussion

Optimization of LC and MS conditions

In order to acquire good separation and peak shapes, the mobile phase system (methanol–water/acetonitrile–water), type of column (BEH C18, HSS T3 and BEH HILIC), column temperature (30, 35, 40 °C) were optimized. The 0.1% formic acid water–acetonitrile were selected as mobile phase system to obtain good distribution for the majority of peaks, HSS T3 column possessed better separation capacity among three different columns, and the column temperature was set 30 °C.

For the MS conditions, both the positive and negative mode were applied for characterization of chemical constituents in HSBDF due to the abundant MS information (Fig. 1). Meanwhile, the capillary voltage, cone voltage and collision energy were optimized, and 2 kV for capillary voltages, 40 V for cone voltage, 15–25 V and 35–45 V were set for collision energy ramp of low mass and high mass.

Characterization of chemical components in HSBDF extract

An UPLC-Q-TOF/MS method was developed for comprehensive characterization of chemical constituents in HSBDF extract in both negative and positive mode. A total of 217 chemical constituents including (alkaloid, flavone, terpenoid, lactones, lignan, phenylpropanoids, phenolic acid, saponin, anthraquinone, others) were tentatively identified based on the MS information, fragmentation pattern of standards and published references, among which 69 chemical constituents were identified in positive mode, and 148 chemi-
Alkaloids were the main bioactive constituents in ephedrae herba, and possessed good therapeutic effect with asthma \(^{[22]}\). Alkaloids had better abundance in positive mode, and the main adduct form was [M + H]\(^+\) peak. Take ephedrine as an example, ephedrine showed an [M + H]\(^+\) ion at m/z 166.12, and fragmented into three products at m/z 148.11, 133.08, 117.06 (Fig. 4). As shown in Fig. 4c, ephedrine generated bond fission of hydroxyl with loss a H\(_2\)O (18 Da), then dehydroxylated-ephedrine was further fragmented to the product ion at m/z 133 and 117 through losing a CH\(_3\) or NH\(_2\)-CH\(_3\) \(^{[23]}\). Thus, four alkaloids from ephedrae herba were identified. Meanwhile, proline and disobutyl phthalate were also identified in negative mode (Fig. 2). Among them, 14 chemical constituents were identified through comparing the retention time and MS spectra of standards. The numbers of flavone and saponin compounds were higher than other compounds, and accounted for 36% and 10% respectively (Fig. 3).

**Components from ephedrae herba**

Chemical constituents were identified in negative mode (Fig. 2). Among them, 14 chemical constituents were identified through comparing the retention time and MS spectra of standards. The numbers of flavone and saponin compounds were higher than other compounds, and accounted for 36% and 10% respectively (Fig. 3).

**Fig. 1** Characterization of chemical constituents in HSBDF. The chemical constituents in HSBDF extract were characterized in both negative and positive mode by UPLC-Q-TOF/MS.

**Fig. 3** Proportion of different types of chemical constituents in HSBDF
detected [24, 25].

Components from astragali radix

There were 13 flavone, 3 other compounds and 9 saponins were supposedly identified in both positive and negative ion mode from astragali radix [26]. In the positive ion mode, flavone compounds exhibited the [M + H+] ion form, calycosin-7-O-β-D-glucoside was used as a case for deduction of fragmentation pattern (Fig. S1). The precursor ion of calycosin-7-O-β-D-glucoside (m/z 447.1277) was observed at 8.32 min, and fragmented into m/z 285 by losing a glucose (C₆H₁₀O₅). The fragment (m/z 285) was further produced three product ions (m/z 270, 253 and 137) with loss of CH₃, CH₂O and C₆H₄O₂ (Fig. S1d). The saponin compounds possessed good response in negative ion mode, and represented [M + COOH] ion form. The fragmentation pattern was showed with astragaloside IV. The precursor ion (m/z 829.4582) was observed at 14.52 min in MS1 and translated into [M – H+] ion form in MS2 (Fig. S2). Meanwhile, the C-3 and C-6 position C-O bonds of [M – H+] ion were broken and generated two characteristic fragments (m/z 621 and m/z 489). The fragment (glucose, m/z 179) was further ruptured and produced m/z 161, 119 and 101 (Fig. S2c) d 101 (Fig. S2e) [30]. According to the fragmentation pattern and published references, 9 saponin compounds from astragali radix were tentatively identified in HSBDF [28-29].

Components from magnoliae officinalis cortex

There were 7 alkaloids, 2 flavones, 3 phenylpropanoids, 12 lignans and 6 other compounds identified from magnoliae officinalis cortex in positive and negative ion mode [30-33]. For the identification of lignans, magnolol and honokiol were used to deduce the fragmentation pattern (Fig. S3). In negative ion mode, magnolol and honokiol presented [M – H+] ion form (m/z 265 and 265), and the main characteristic fragments were m/z 247 and 224 with loss of OH and C₆H₅ [32]. Furthermore, 7 alkaloids were supposedly identified on the basis of the published references [30-34].

Components from armeniacae semen amaran and glycyrrhizae radix et rhizoma

Two compounds (amygdaalin and prunasin) were identified from xingren. The MS1, MS2 and fragmentation pattern of amygdaalin were shown in Fig. S4. A total of 20 flavones, 13 saponins, 1 disaccharide and 1 phenylpropanoids were found from gancao. One flavone (liquiritin) and saponin (glycyrrhizic acid) were applied for deduction of fragmentation pattern. For the liquiritin, the glycoside was lost from [M – H+] ion (m/z 417) and the m/z 255 was further fragmented into m/z 135 and 119 (Fig. S5). For the saponin, the precursor ion was [M + H+] ion at m/z 821, and the main fragment ions (m/z 645 [M – H-(C₆H₅O₃)] , m/z 351 [2C₆H₅O₄ – H]+, m/z 195 [C₆H₅O₄–] ) that were generated via the MS/MS fragmentation were observed in the negative ion spectrum [33].

Components from rhei radix et rhizoma

Forty compounds including 7 phenolic acids, 7 flavonoids, 14 anthraquinones, 3 phenylpropanoids and 8 other compounds from rhei radix et rhizoma were tentatively identified in negative ion mode [36-37]. For the anthraquinones, emodin and rhein were used as examples to illustrate the characterization process. The characteristic fragments (m/z 241 and 225) were fractured from [M – H+] ion (emodin) at m/z 821 by losing CO and OH (Fig. S6). The [M – H+] ion of rhein was presented at m/z 283 in MS1, and the fragments (m/z 239, 211 and 183) were produced with loss of COOH, CO and CO in MS2 (Fig. S7). The phenolic acids, flavones and phenylpropanoids were supposedly characterized through comparing with elementary composition and characteristic fragments derived from published references [38-39].

Components from descurainiae semen lepidii semen

There were 6 flavones from descurainiae semen lepidii semen were tentatively identified under negative ion mode in HSBDF [40-41]. Quercetin-3-O-β-D-glucoside-7-O-β-gentiobioside showed [M – H+] form at m/z 787, and three fragments (m/z 625 [M – H-glc], m/z 463 [M – H-2glc], and m/z 301 [M – H-3glc] ) were observed in MS2 (Fig. S8). The fragmentation pattern of quercetin-3-O-β-D-glucoside-7-O-β-gentiobioside was exhibited in Fig. S8c.

Components from paeoniae radix rubra

A total of 14 compounds (2 phenolic acids, 2 flavones and 11 terpenoids) from paeoniae radix rubra were tentatively identified in HSBDF [52-61]. Paeoniflorin was used as a case for elucidation of proposed fragmentation pathways of terpenoids. As shown in Fig. S9, the precursor ion was [M + COOH] form at m/z 525 in MS1, and three fragments (m/z 449, 327 and 121) were produced in MS2. The m/z 449 was generated by losing a HCHO (30Da), and further fragmented into a minor fragment ion at m/z 327 and a benzoic acid ([M – H], 121 Da). According to the fragmentation pattern, 11 terpenoids were tentatively characterized in HSBDF [44-47].

Components from agastache rugosus, atractylodis rhizoma, tsacao fructus and pinelliae rhizoma praeparatum

There were 17 compounds (1 lignan, 4 phenylpropanoids and 12 flavones) from agastache rugosus [44-51] and 26 compounds (4 terpenoids, 3 flavones, 5 lactones, 1 phenylpropanoid and 13 other compounds) from atractylodis rhizoma [52], 8 compounds (3 phenolic acids and 5 flavonoids) from tsacao fructus [53], 8 compounds (4 flavone and 4 other compounds) from pinelliae rhizoma praeparatum were supposedly identified in HSBDF [55-56].

Cytotoxicity assay in RAW264.7 cells

We evaluated cell viability of RAW264.7 cells cultured with different concentrations of HSBDF using the MTT assay to find a suitable concentration for application in the subsequent experiment. These results indicated that the HSBDF showed unapparent cytotoxicity to RAW264.7 cells at concentrations up to 200 μg·mL⁻¹. The CC₅₀ of HSBDF toward RAW264.7 cells was 417 μg·mL⁻¹ (Fig. 5).
Inhibition of the production of TNF-α and IL-6 in LPS-induced RAW 264.7 macrophages

Severe acute respiratory syndrome CoV (SARS-CoV) and Middle East respiratory syndrome CoV (MERS-CoV) were often associated with massive inflammatory cell infiltration and elevated proinflammatory cytokine/chemokine responses resulting in severe respiratory illness. To determine the effect of HSBDF on the expression of proinflammatory cytokine, an in vitro LPS-stimulated inflammatory response cell model was established. The expression levels of IL-6 and TNF-α in the cell supernatant were measured by ELISA. As shown in Fig. 6, the elevated expressions of these cytokines induced by LPS in RAW264.7 cells were inhibited by HSBDF treatment in a concentration-dependent manner (*P < 0.05).

![Figure 6](https://example.com/figure6.png)

**Fig. 6** Effects of HSBDF treatment on the expression levels of inflammatory cytokine in LPS-stimulated RAW264.7 cells via ELISA test. (A) IL-6; (B) TNF-α. Data are presented as mean ± SD (n = 3), *P < 0.05 vs blank group.

**Conclusion**

In this study, a rapid method was developed and applied to characterize and profile the chemical components in HSBDF using UPLC-Q-TOF/MS. A total of 217 constituents including alkaloids, flavones, terpenoids, lactones, lignans, phenylpropanoids, phenolic acids, saponins, anthraquinones and other compounds were identified or tentatively characterized. It was known that COVID-19 infection could induce immediate and late host immune responses in patients. Most severe COVID-19 cases exhibited an extreme increase in inflammatory cytokines, including IL-1β, IL-6, IL-10, IFN-γ, and TNF-α, representing a “cytokine storm”. Though the precise mechanism of antigen presentation, cellular and humoral immune responses, and cytokine storm during the COVID-19 infection were not yet clearly understood, but the increasing level of IL-6 in COVID-19 patients could induce the differentiation of proinflammatory Th17 cells, upregulate cytokine storm, lung inflammation and dysfunction. Thus proinflammatory cytokine expression levels (IL-6 and TNF-α) upon LPS administration in RAW264.7 cells were measured and results were shown that HSBDF could alleviate the expression levels of IL-6 and TNF-α in the cell models, indicating that the antiviral effects of HSBDF might be attributed to the regulation of the inflammatory cytokines production. It is hoped that the results could provide the essential data for the further study on pharmacodynamic material basis of HSBDF in COVID-19.

**Supplementary Materials**

Supplementary materials are available as Supporting Information, and can be requested by sending E-mail to the corresponding author.

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