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Comparison of bioactive components and pharmacological activities of *ophiopogon japonicas* extracts from different geographical origins

Min Zhao[a,1], Wan-feng Xu[a,1], Han-yuan Shen[a], Pei-qiang Shen[b], Jun Zhang[a], Dan-dan Wang[a], Han Xu[b], Hong Wang[a], Ting-ting Yan[a], Lin Wang[a], Hai-ping Hao[a], Guang-ji Wang[a,*], Li-juan Cao[a,*]

[a] *State Key Laboratory of Natural Medicines, Key Lab of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, Tongjiaxiang 24, Nanjing, 21009, China*
[b] *ChiaTai Qingchunbao Pharmaceutical Co. Ltd., Xixi Road 551, Xihu District, Hangzhou, China*

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ABSTRACT

Ophiopogon japonicus (Linn. f.) Ker-Gawl (*O. japonicas*), mainly cultivated in Sichuan and Zhejiang province in China, has different bioactive components and therefore their pharmacological activities. To explain the different clinical efficacy of *O. japonicas* derived preparations, herein we report differences of pharmacological activities between Sichuan and Zhejiang *O. japonicas* and behind them the exact differences of bioactive components. Based on a LC/MS-IT-TOF method, the differences of bioactive components between Sichuan and Zhejiang *O. japonicas* extracts were analyzed and respective characteristic components were picked out. We determined 39 ophiopogonones and 71 ophiopogonins compounds in Sichuan and Zhejiang *O. japonicas* extracts and found the contents of these compositions have several times difference. Evidenced by experimental data of pharmacological activities in inhibiting cardiomyocyte damage induced by H_2O_2, mouse macrophage cell inflammation induced by lipopolysaccharide and cytotoxicity *in vitro*, Zhejiang *O. japonicas* extract had a stronger antioxidant and anti-inflammatory capacity than Sichuan *O. japonicas* extract, and the two *O. japonicas* extracts exhibited selective cytotoxicity on different cancer cell lines *in vitro*. These data shed light on the links between bioactive components and pharmacological activities of *O. japonicas* derived preparations. Thus, geographical origin of *O. japonicas* should be considered to be a key factor in efficacy studies and further clinical application.

1. Introduction

Ophiopogon japonicus (Linn. f.) Ker-Gawl (*O. japonicas*, Maidong in Chinese) is widely distributed and used in East Asia, especially in China [1]. *O. japonicas* is used in many compound prescriptions, such as Shenmai injection, Maiwei pill, Shengmai capsule and Xuanmai granule, etc. The main pharmacological activities of *O. japonicas* are to moisturize lung by nourishing Yin, replenish Qi and purge heat referring to traditional Chinese medicine's theory. Ophiopogonis radices, dried tubers of *O. japonicas* are routinely used as traditional Chinese materials whose extract (*O. japonicas* extract) exhibits various pharmacological activities, such as cardiovascular protection, anti-cancer, anti-oxidation, immunomodulation, cough relief, antimicrobial and anti-diabetes [2–6].

Approximately 75 steroidal saponins, 36 homoisoflavonoids, 11 polysaccharides and 13 organic acids have been isolated and identified from *O. japonicas* through a variety of methods [7]. With the power of TOF/MS, more than 50 ophiopogonins and 27 ophiopogonones in *O. japonicas* extract were simultaneously detected and identified using an approach of mass defect filtering (MDF) as reported in our previous work [8]. The main active components in *O. japonicas* include ophiopogonones, ophiopogonins and polysaccharides [2,5,9], among which ophiopogonones and

Abbreviations: *O. japonicas*, Ophiopogon japonicus (Linn. f.) Ker-Gawl; LPS, lipopolysaccharide; LC/MS-IT-TOF, high performance liquid chromatography coupled with hybrid ion trap and time-of-flight mass spectrometry; MDF, mass defect filtering; CCK-8, cell counting kit 8; LDH, hactate dehydrogenase; NAC, N-acetylcysteine; ROS, Reactive oxygen species; MDA, Malondialdehyde; IL-1β, interleukin 1βa; IL-6, interleukin 6; IL-18, interleukin 18; TNF-α, tumor necrosis factor α; NLRP3, NACHT LRR and PYD domains-containing protein 3; PCA, Principal Component Analysis; VIP, variable importance in projection; PLS-DA, partial least squares discriminant analysis; CI, confidence interval.

* Corresponding authors.
E-mail addresses: guangjiwang@hotmail.com (G.-j. Wang), caolijuan0702@cpu.edu.cn (L.-j. Cao).
[1] These authors contributed equally to this work.

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ophiopogonins are most important components attributing to the efficacy of *O. japonicas*. Ophiopogonones components are mainly responsible for its anti-oxidation and anti-inflammatory efficacies, for examples, ophiopogonanone H and other 13 ophiopogonones are able to inhibit NO production induced by lipopolysaccharide (LPS) [10], Ophiopogonones derived compounds can inhibit the release of the inflammatory chemokine eotaxin [11]. Ophiopogonins components are particularly effective in anti-cancer and immunomodulation, for instance, both ophiopogonin B and ophiopogonin D can effectively inhibit cell proliferation and induces apoptosis [12], steroidal ophiopogonins shows selective cytotoxicity against different cell lines [13,14], and ruscogenin exerts anti-inflammatory activity by suppressing the expression of ICAM-1 and NF-κB pathway [15,16].

Shenmai injection is one of the most widely used *O. japonicas* extract preparations in clinical application in China. It has been proved to improve New York Heart Association (NYHA) functional classification for patients with chronic heart failure and coronary artery disease [17]. *O. japonicas* is mainly cultivated in Sichuan and Zhejiang province in China, they possess different saponin contents and homoisoflavonoids components and related antioxidant activities [18,19]. Zhejiang *O. japonicas* extract exhibits a significant higher cytotoxicity, stronger induction of CYP3A4 and relatively weaker activation of PXR expression than Sichuan *O. japonicas* extract [20]. However, the all-round differences of components and pharmacological activities between Sichuan and Zhejiang *O. japonicas* remains largely elusive. Hitherto, differences between Sichuan and Zhejiang *O. japonicas* extracts are largely confined to single components, such as ophiopogonin B, D [18,19], while lack of a comprehensive composition comparison between them, and few reports has elucidated the global component differences of extracts from different geographical origin, neither related different pharmacological properties.

In light of the above, this paper was designed aiming to analyze the overall differences of ophiopogonones and ophiopogonins in Sichuan and Zhejiang *O. japonicas* extracts and associate their bioactive components to pharmacological activities.

2. Materials and methods

2.1. Materials, reagents and chemicals

Sichuan and Zhejiang *O. japonicas* were obtained from ChiaTai Qingchunbao Pharmaceutical Co., Ltd. (Zhejiang, China). Formic acid (Cat.56302) and H_2O_2 (Cat.88597) were purchased from Sigma-Aldrich (Shanghai, China). Cell Counting Kit-8 (CCK-8) was purchased from DOJINDO (Kumamoto, Japan). Lactate dehydrogenase (LDH), Reactive oxygen species (ROS), Malondialdehyde (MDA) detection kits, RIPA lysis buffer, BCA Protein Assay Kit and N-acetylcysteine (NAC) were purchased from Beyotime (Shanghai, China). HPLC grade acetonitrile was obtained from Merck (Damstadt, Germany). All aqueous solutions were prepared with deionized water purified by a Milli-Q Ultrapure water system (Millipore, Bedford, USA).

2.2. Extracts preparation

200 g *O. japonicas* were cut into pieces and soaked with 600 ml 75% ethanol for 15 min at 20 °C, then the mixture was heat reflux extracted for 2 h twice in 600 ml 75% ethanol and the ethanol extract was collected, condensed to 600 ml under reduced pressure and sub-packed in 20 ml ampoules. For LC/MS analysis and cell experiments, extracts were lyophilized and re-dissolved with equal volume of methanol (for LC/MS analysis) or RMPI 1640, DMEM and McCoy's 5A medium (for cell experiments).

Table 1
Gradient elution process.

t [11]	5	30	33.5	58.5	61	62.5	69.5
B%	23	40	40	80	80	23	stop

2.3. Chromatography and mass parameter settings

LC/MS-IT-TOF (including LC/MS Solution chromatography workstation, Profile Solution Program software, Shimadzu Corporation). Chromatography parameters: Column of Agilent ZoBax SB-C18, 2.1 × 150 mm i.d., 3.5 μm (Agilent Technologies, USA), the column temperature was 40 °C, flow rate: 0.2 ml/min, the mobile phase consists of deionized water (containing 0.02% formic acid, A) and acetonitrile (B), The injection volume was 5 μl, gradient elution was shown in Table 1. Data were represented as the peak area ratio to internal standard (1.0 μg/ml of digoxin).

Mass spectrometry conditions: electrospray ionization mode (Electronic spray ionization, ESI); negative ions. Mode subscan analysis; ophiopogonins scan range: MS1 *m/z* 500–1500, MS2 *m/z* 100–800, MS3 *m/z* 50–600; multistage fragmentation collision energy: MS2 and MS3 located respectively 50% and 100%; ophiopogonone scan range: MS1 *m/z* 200–500, MS2 *m/z* 100–300, MS3 *m/z* 50–200, multi-level fragmentation collision energy: MS2 and MS3 Located respectively 50% and 100%; detection voltage: 1.60 kV; atomizer gas flow (N2): 1.5 l/min; drying gas flow (N_2): 5 l/min; Curved solvent removal device (CDL) and the heating block (block) temperatures are 200 °C, collision gas is high pure argon gas (purity: 99.999%); TOF degree of vacuum: 1.5×10^{-4} Pa; ion trap degree of vacuum: 1.7×10^{-2} Pa; Ion Accumulation Time: 30 ms; accurate mass calibration using trifluoroacetic acid sodium.

2.4. Compound prediction

The characteristic peaks of two *O. japonicas* extracts were compared to methanol and pick out using Profile Solution Program software, via subtracting methanol background, which peak areas greater than 1×10^7 were considered as effective substances. Compound prediction was carried out by MDF strategy established previously [8]. To be detail, ophiopogonones in *O. japonicas* can be distinguished as homoisoflavone and homoisoflavanone based on the saturation between C2 and C3 in ophiopogonone nucleus. The connection between C2 and C3 of homoisoflavone is a double bond and its fragmentation characteristics is a neutral loss of 13 Da. For aldehyde substituted homoisoflavone, it will produce a characteristic neutral loss of 54 Da. The connection between C2 and C3 of homoisoflavanone is a single bond and its fragmentation characteristics is a neutral loss of 28 Da. According to diagnostic ions extension strategy for ophiopogonin identification, both ionization behavior of co-exist of $[M-H]^-$ and $[M+HCOOH-H]^-$ and neutral loss of carbohydrate are the unique properties of ophiopogonin, which can provide references for compound searching and confirmation. After sorted out the information in different fragmentation energy, we predicted molecular formula of fragments and searched for compounds in our compound library [8] and the existing database (Dictionary of Natural Products, Chapman & Hall/CRC Combined Chemical Dictionary, etc.)

2.5. Cell culture

Rat cardiomyocyte cell line H9c2, mouse macrophage cell line Raw 264.7, non-small cell lung cancer line A549, human hepatoma cell line HepG2, human breast cancer cell line MCF-7/S, human colon cancer cell lines HCT116 and HT 29 were obtained from American Type Culture Collection (ATCC, Virginia, USA). H9c2, Raw 264.7 and HepG2 cells were grown in DMEM medium, A549 cells