Screening and Identifying Hepatotoxic Components in Polygoni multiflori Radix and Polygoni multiflori Radix Praeparata

Guang-Ping Zhang, Hai-Jing Zhang, Teng-Fei Chen, Hong-Ping Hou, Ping Su, Yun-Hang Gao, Yi-Fei Yang, Zu-Guang Ye

Abstract

Objective: In this study, the hepatotoxic components of Polygoni multiflori Radix and Polygoni multiflori Radix Praeparata (known as Heshouwu [HSW] and Zhiheshouwu [ZHSW] in China, respectively) were screened, isolated, and identified. Materials and Methods: The ethanol extracts of HSW and ZHSW were separated into 80 fractions according to their polarity in the preparation liquid phase. Chang liver cell line was used to screen the toxic components of HSW and ZHSW in vitro. The obtained toxic mixture was further collected, isolated, and identified to confirm the hepatotoxic compounds of HSW and ZHSW. Results: The identified hepatotoxic compounds include 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside, emodin, physcion-8-O-β-D-glucoside, physcion, and citreorosein, the first two among them were the main identified to confirm the hepatotoxic compounds of HSW and ZHSW. After processing of HSW, the contents of 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside and emodin in ZHSW were significantly decreased. Conclusions: The traditional processing with herb has significant effects on the components, especially the toxic components, in the extract of HSW and is an effective method to reduce its toxicity.

Keywords: 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside, emodin, hepatotoxicity, Polygoni multiflori Radix, Polygoni multiflori Radix Praeparata

INTRODUCTION

Polygoni multiflori Radix, also known as Heshouwu (HSW) in China, is the dried root of Polygonum multiflorum Thunb. As a traditional tonic medicine, it has been used for thousands of years in clinical practice.[1] The processed form of HSW was named as Polygoni multiflori Radix Praeparata (also known as Zhiheshouwu [ZHSW]), which is boiled in black bean liquid according to a traditional process. The two forms have different efficacy: the HSW is used for detoxification, eliminating carbuncle, preventing malaria, and relaxing bowel, whereas the ZHSW is used for nourishing liver and kidney, supplementing essence and blood, blackening hair, strengthening bones and muscles, and used as tonic functional foods.[2]

Consumers have usually believed that HSW is relatively safe because of their natural resource and traditional applications. However, the first recognized case of hepatitis caused by proprietary Chinese medicines in Hong Kong is a case of acute hepatitis associated with Shou-wu-Pian.[3] Then, the hepatotoxicity of HSW was reported in 2001 in Australia[4] and in 2006 in the United Kingdom by the Medicines and Healthcare products Regulatory Agency.[5] After that, follow-up reports on adverse hepatotoxic effects caused by HSW or its constituents have increased worldwide. Recently, the safety of HSW has attracted widespread concern in the world, and its supervised usage is recommended by various countries.[6,7]

Due to the complex components and multiple functions of HSW, its liver toxicological substances are difficult to be defined, and the mechanisms are still unclear. The case report from Hong Kong speculated that the herb’s hepatotoxicity may be attributable to the presence of anthraquinones.[3,4] While Wu et al. found that the main toxicants in HSW do not depend on the content of anthraquinone derivatives, it may be correlated with the content

Address for correspondence: Prof. Zu-Guang Ye, Institute of Chinese Materia Medica, No. 4, Sakura East Road, Chaoyang District, Beijing 100700, China. E-mail: zgye@icmm.ac.cn

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of tetrahydroxystilbene glucosides.\textsuperscript{[3]} However, some studies have shown that the ethanol extract of HSW had hepatotoxicity, while the water extracts of HSW elicited beneficial effects on mice liver without any toxicity and can ameliorate the liver damage caused by CCl\textsubscript{4}.\textsuperscript{[9-11]} To clarify the hepatotoxic compounds and the feasibility of processing to reduce the toxicity of HSW, we screened, isolated, and identified the hepatotoxic components of ethanol extracts of HSW and ZHSW and compared the content of these two extracts. We hope that our study will provide scientific basis for guiding the rational clinical use of HSW to reduce the occurrence of hepatotoxicity in the future.

**Materials and Methods**

**Chemicals and reagents**

Chang liver cell line was provided by the Central Laboratory of the Second Hospital of Jilin University (Jilin China). HSW and ZHSW were purchased from the Tongrentang (Beijing, China). High-performance liquid chromatography-mass spectrometry (HPLC-MS) grade acetonitrile and methanol and Dulbecco’s modified Eagle’s medium (DMEM) were supplied by the Sigma-Aldrich (MO, USA). Fetal bovine serum (FBS) for cell culture was obtained from HyClone (UT, USA) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation, and cytotoxicity test kit (C0009) was ordered from Biyuntian Biotechnology Co., Ltd (Shanghai, China).

**Instruments**

The Waters 2695 Alliance systems (Waters, Milford, MA, USA) was used to analyze; this system consisted of Waters 2996 multiwavelength visible ultraviolet detector and Waters 2525 binary high-pressure gradient pump. A preparative HPLC system (Waters, Milford, MA, USA) was used for the preparation of fractions and the isolation of individual compounds. This system consisted of Waters 2478 dual-wavelength ultraviolet detector and Waters 2767 stream collector.

**Separation and preparation of extractum fractions of Heshouwu and Zhiheshouwu**

The fully-dried HSW and ZHSW were pulverized and sifted through a 100-mesh sieve. The fine powder (30 g) of HSW and ZHSW was extracted two times with 95% ethanol for 12 h using Soxhlet extractor, respectively. The two extracting solutions were combined and filtrated to remove the particulate impurities. The filtrate was vacuum evaporated to recover the solvent. The extractum was stored at −20°C.

The above extractum of HSW and ZHSW was dissolved in 80% methanol and preliminarily separated using preparative HPLC with a Xterra C\textsubscript{18} chromatographic column (100 mm i.d. × 19 mm, 5 μm) which obtained from Waters (MO, USA). Mobile Phase A was methanol, and mobile phase B was water. The gradient elution program of mobile phase was as follows: 0–30 min, 10% ~ 80% A; 30–40 min, 80% ~ 100% A. The flow rate was 10 mL/min, the column temperature was 30°C, the injection volume was 800 μL, and the detection wavelengths were 200 nm and 254 nm. The extractum of HSW and ZHSW was collected in 80 fractions, respectively.

**Cell culture and screen of toxic extractum fractions of Heshouwu and Zhiheshouwu**

The measurement of cell cytotoxicity was performed on Chang liver cell line which was cultured in DMEM (containing 10% FBS, 1% GlutaMAX, 100 μg/mL penicillin and streptomycin) and kept at 37°C and 5% CO\textsubscript{2} in cell incubator. The culture medium was replaced every 48 h. The cells were planted in 96-well plates at the density of 3 × 10\textsuperscript{4} cells/mL and allowed to adhere for 24 h. After that, the cells were respectively treated with different fractions of HSW or ZHSW for 48 h. After incubated for 4 h at 37°C with MTT, the cell viability was determined colorimetrically by absorbance at 570 nm. The absorbance values of medium-treated cells (negative control) were standardized as 100%, and the viability values of test samples were expressed as percentage of negative control.

**Analytical of toxic extractum fractions of Heshouwu and Zhiheshouwu**

The toxic extract fractions (F5–F8) analysis was performed on a Waters Alliance 2695 HPLC system. Mobile Phase A was acetonitrile, mobile Phase B was 0.03% trifluoroacetic acid solution (v/v). The gradient elution program of mobile phase was as follows: 0–5 min, 10% A; 5–15 min, 10% ~ 30% A; 15–35 min, 30% A; 35–45 min, 30% ~ 100% A; 45–50 min, 100% A; 50–55 min, 100% ~ 10% A; and 55–70 min, 10% A.

**Activity-induced separation of toxic compounds**

**Preparative of toxic parts**

The preparation of toxic parts was described as follow: the extractum of HSW (12 g) was dissolved in methanol-dichloromethane (1:1, v/v), stirred in two times amount of silica gel. Then, the...
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Separation of toxic compounds
After further optimization of the analytical method, certain chromatographic conditions were selected to separate the main components of HSW-16 fraction through semi-prepared liquid phase. The mobile phase was: A: 100% acetonitrile and B: 10% acetonitrile-water with 0.03% trifluoroacetic acid (v/v). The chromatographic separation conditions were: 33%A/67%B, time: 120min, flow rate: 10 mL/min. The main chromatographic peaks in the analysis process were enriched, evaporated and dried. Finally, three monomer compounds were isolated and purified, named HSW-F9-1, HSW-F9-3 and HSW-F10.

Structure elucidation of the isolated compounds
For the five monomeric compounds, 1 mg was extracted and dissolved in methanol, and then AB SCIEX API 3200 LC-MS/MS was used for identification. The MS conditions were as follows: in the anion mode, declustering potential (DP): −60, entrance potential (EP): −10, Q1 scanning range was m/z 200–500, and the time was 0.2 s in the positive ion mode; DP: 55, EP: 10, Q1 scanning range was m/z 200–500, and the time was 0.2 s. In addition, the samples (5 mg) were dissolved in dimethyl sulfoxide-d6 solvent. The proton nuclear (1H) and 13C-nuclear magnetic resonance (C-NMR) analyses were performed on Inova-500M NMR instrument (Varian, USA) at 22°C. The chemical shifts are based on tetramethylsilane as standard.

Results
Screening of the toxic fractions in ethanol extracts of Heshouwu and Zhiheshouwu
The extractum of HSW and ZHSW was dissolved in 80% methanol and preliminarily separated into 80 fractions (F1 ~ F80) using preparative HPLC, respectively. After drying, the samples were dissolved in dimethyl sulfoxide (DMSO), respectively, according to the average molecular weight of potential toxic parts were separated and purified by medium pressure liquid chromatography (LC) and octadecyl-silica column. mobile phase A was methanol-water (10:90, v/v) with 0.03% trifluoroacetic acid (v/v), mobile phase B was 100% methanol. The gradient elution program of mobile phase was that: 0–180 min, 90% ~ 0% A with the flow rate at 10 mL/min. The fractions were collected starting from the peak to obtain 12 parts. Each part was evaporated and redissolved in the mobile phase, and the toxic parts were compared with those screened before by the analytical liquid phase. Finally, four parts named HSW-11, HSW-16, HSW-C, and HSW-X were determined to be the main toxic parts.
500 g/mol to obtain the mother liquor of each component (20 mM). Herein, the toxicities of 80 fractions of HSW and ZHSW on Chang liver cell line were investigated by the MTT assay with the final concentration of 200 µM. The results showed that the treated cells with fraction F5 ~ F8 of HSW resulted in 60.54% ~ 67.94% inhibition of the cell viability at 24 h, while the fraction F5 ~ F8 of ZHSW had slight cytotoxicity (8.67% ~ 34.67% inhibition of the cell viability) [Figure 1].

**Identification of the toxic components**

The screened toxic fractions (F5 ~ F8) of HSW were analyzed by analytical HPLC. The results are shown in Figure 2, F5 ~ F8 fractions contain the same component, and the main components are compounds with a retention time of 36 min.

The same chromatographic characteristics are also shown in ZHSW. The results are shown in Figure 2.

**Preparation of the toxic fractions**

For preparation of toxic fractions from ethanol extracts of HSW, the fractions were collected starting from the peak to obtain 12 fractions. Finally, it was determined that the four fractions (HSW-11, HSW-16, HSW-C, and HSW-X) were the main toxic sites by comparing with the previously sifted toxic components. The results were shown in Figure 3.

**Revalidation of the toxic fractions**

The powders prepared from the above four medium-pressure fractions (HSW-11, HSW-16, HSW-C, and HSW-X) were dissolved in DMSO to produce samples with concentrations

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**Figure 5:** The nuclear magnetic resonance spectra and structures of Heshouwu-11 and Heshouwu-C. (a) 1H Proton nuclear-nuclear magnetic resonance (500 MHz, dimethyl sulfoxide-d6) spectra of Heshouwu-11; (b) 13C Carbon-nuclear magnetic resonance (125 MHz, dimethyl sulfoxide-d6) spectra of Heshouwu-11; (c) The chemical formula for Heshouwu-11; (d) 1H Proton nuclear-nuclear magnetic resonance (500 MHz, dimethyl sulfoxide-d6) spectra of Heshouwu-C; and (e) 13C Carbon-nuclear magnetic resonance (125 MHz, dimethyl sulfoxide-d6) spectra of Heshouwu-C; (f) The chemical formula for Heshouwu-C
of 0, 200, 400, and 800 µM, respectively. The samples were added to the cell plate and then tested by MTT assay. The results showed that the four fractions had certain cytotoxicity, and the toxicity of the four components from strong to weak is HSW-16, HSW-C, HSW-11, and HSW-X [Figure 4].

**Identification of the isolated compounds**

On the basis of the above screening results, the isolation and purification of toxic compounds were performed on the preparative HPLC system to obtain five monomeric compounds. Among them, HSW-11 and HSW-C were monomer components with a relative purity of 98%. HSW-16 was eluted with a gradient program to afford three monomeric compounds, namely HSW-F9-1, HSW-F9-3, and HSW-F10. Five compounds were obtained at high purity from three toxic fractions. Identification of the pure compounds was performed by 1H and 13C-NMR. By means of the spectral data analysis, combined with previous reports, HSW-11, HSW-C, HSW-F9-1,
HSW-F9-3, and HSW-F10 were identified as 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucopyranoside, emodin, physcion-8-O-β-D-glucopyranoside, citreorosein, and physcion, respectively. The structures of the isolated compounds and their NMR spectra were shown in Figures 5–7.

**Determination of toxic substances in ethanol extracts of Heshouwu and Zhiheshouwu**

To further study, the difference in the content of the ingredients before and after processing, we separately analyzed the HPLC of HSW and ZHSW. As shown in Figure 8, the compounds HSW-11 (2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucopyranoside) and HSW-C (emodin) were the main components in the extract of HSW, and the contents of these two compounds after processing were significantly reduced by about 477 and 112 times, respectively, while the MTT assay showed that HSW-11 and HSW-c had obvious hepatocytotoxicity.

**Discussion**

HSW and ZHSW are both traditional Chinese medicinal herb in the Chinese Pharmacopoeia. For traditional efficacies, HSW is used for detoxification, eliminating carbuncle, preventing malaria, and relaxing bowel, while ZHSW is a steamed preparation of HSW with black bean and is used for nourishing liver and kidney, supplementing essence and blood, blackening hair, strengthening sinews and bone. In addition, both drug forms could lead to drug-induced liver injury and even death in vitro, in vivo, and in clinical. Since HSW exhibited both pharmacological functions and hepatotoxic effects, it is essential to determine the toxic compounds and evaluate the safety when it is in clinical use.

As to the toxicity in vivo studies, the literature shows that the ethanol extract of HSW tends to be more toxic than water extract and acetone extract, therefore, our study selected the ethanol extracts for subsequent screening of toxic substances. Five main toxic compounds have been screened out and identified as 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside, emodin, citreorosein, physcion, and physcion-8-O-β-D-glucoside. Since 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside and emodin were the main components of HSW, we confirmed these two compounds as the key indicators of hepatotoxicity. Our results were basically similar to the research reported in the literature. Previous studies found that the main toxic compounds of HSW may dominantly be attributed to its components of anthraquinones and tetrahydroxystilbene glucosides. HSW is more toxic than ZHSW, and the higher concentration of ethanol extract shows more toxicity. Our findings also confirm this statement. Unlike HSW, the contents of 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside and emodin in ZHSW were significantly reduced by about 477 and 112 times, respectively. It was suggested that processing can reduce the toxicity of HSW obviously. Furthermore, both HSW and ZHSW have toxic effects on liver, and the toxic compounds for this effect are 2,3,5,4-tetrahydroxystilbene-2-O-β-D-glucoside and emodin. On the other hand, both HSW and ZHSW protect liver cells from nonalcoholic fatty liver disease, oxidation, fibrosis, cirrhosis, and liver cancer, and the active compounds for this effect are 2,3,5,4-tetrahydroxystilbene-2-O-β-D-glucoside, emodin, and physcion. This suggests that the key to the hepatoprotection or hepatotoxicity of HSW is the dose, and rational use of drugs can effectively avoid hepatotoxic effects.

Some HSW-induced liver injury may be predictable based on the pharmacodynamic and pharmacokinetic properties. They are completely safe over a wide range of doses for the vast majority.
of treated patients, but severely toxic to a small subset of patients. This low incidence and unpredictability phenomenon may be a response to complex genetic regulatory disorders caused by a combination of in vitro factors and in vivo polygenic variants.[24]

Conclusions

The traditional processing with herb has significant effects on the components, (especially the toxic components) in the extract of HSW. The hepatotoxic components including 2,3,5,4’-tetrahydroxystilbene-2-O-β-D-glucopyranoside, emodin, citreorosein, physcion, and physcion-8-O-β-D-glucopyranoside have been screened and validated, and the first two are the main components of HSW. The results showed that, after processing of HSW, the contents of 2,3,5,4’-tetrahydroxystilbene-2-O-β-D-glucopyranoside and emodin were significantly reduced. It was proved that processing of HSW was an effective method to reduce its toxicity.

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Conflicts of interest

There are no conflicts of interest.

References