Spectrum-Toxicity Correlation Study Revealed the Influence of the Nine-Time Steaming and Sun Drying Method on Hepatotoxic Components of Polygoni Multiflori Radix

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Objective: Polygoni Multiflori Radix (PM) is a traditional herbal medicine with repeated reports of liver injury events in recent years. We wondered whether the classical processing method, namely, nine-time steaming and sun drying (NSSD), had toxicity-attenuating effects on PM and the relationships between toxicity and times of processing, as well as with the alteration trends of its compounds.

Materials and Methods: The chemical fingerprints of different PM extracts were developed using ultra-high-performance liquid chromatography. The spectrum-toxicity correlation between the chemical fingerprints and hepatocellular toxicity was analyzed with multiple correlation analysis.

Results: The results suggested that the hepatotoxicity of NSSD processing products markedly decreased with the repeated steaming and sun drying, which was obviously superior to the product processed by the modern method. Comprehensive analysis revealed that the contents of cis-stilbene glycoside and emodin-8-O-β-D glucoside related to liver injury susceptibility were reduced with the times of NSSD processing, which was consistent with the decreased trend of hepatocellular toxicity. After the five times of NSSD, the contents of them as well as the hepatotoxicity of PM were steady. Moreover, we found that the contents of catechin and physcion declined rapidly after the one time of NSSD and then remained stable until the nine times of NSSD. Based on the fact, they could be utilized to indicate whether PM products were processed by steaming and sun drying. Conclusions: This paper confirmed that the NSSD had a good influence on the toxicity attenuating to PM and found four compounds which could apply for the quality control of PM.

Keywords: Hepatotoxicity, nine-time steaming and sun drying, polygoni multiflori radix, spectrum-toxicity correlation, toxicity attenuation by processing

Introduction

Polygonum multiflorum Radix (PM) is the dry root of Polygonum multiflorum Thunb., a kind of traditional herb with a long history of medication in China. Crude PM has the function of detoxification, pain relief, and laxative, while its processed products are considered as nontoxic herbs which are routinely used for tonifying liver and kidney, benefiting vital energy, and preventing the premature graying of hair. [1-4] Due to the increasing reports on PM-induced liver injury, it has aroused extensive attention worldwide. [5-11] Based on the constituent knock-out and knock-in strategy, our previous studies found that two susceptibility-related compounds of PM, cis-stilbene glycoside (CSG) and emodin-8-O-β-D-glucoside (EG), mainly contributed to hepatotoxicity. [12,13] However, it remained unclear...
about the alteration trends of these components during the processing of PM.

Moreover, there were few mentions of the toxicity of PM in ancient medical masterpieces, where often recorded a classical processing method, the nine-time steaming and sun drying (NSSD). A famous ancient medical book, called *Materia Medica compile (Ben Cao Hui Yan)*, expounded that the crude PM was of cold characteristic and toxic, while its preparation was mild and nontoxic, which clearly put forward the toxicity attenuation of PM by processing. The modern processing method of PM, which included in the *People’s Republic of China Pharmacopoeia* (2015 edition), was steaming with black soybean liquid without requirements for repeated steaming or sun drying. We doubted whether the NSSD method had a different influence on the hepatotoxicity of PM and wondered about the relationships between toxicity and times of processing. In this study, we established the spectrum-toxicity correlation to compare the NSSD with the modern method, then screened and confirmed major hepatotoxic components of PM. This study will provide data support for further hepatotoxicity research and facilitate improvement on processing methods as well as quality control of PM.

**Materials and Methods**

**Reagents and materials**

Polygonum multiflorum Radix (PM) was purchased from Beijing Wizard Pharmaceutical Co. Ltd., China, and identified as the root of *P. multiflorum* Thunb. by Prof. Xiao-he Xiao, General Hospital of PLA. Black soybean was purchased from Beijing Wizard Pharmaceutical Co. Ltd. and identified as the mature seeds of *Glycine max* (L.) Merr. RPMI-1640, fetal bovine serum (FBS), 0.25% trypsin, and streptomycin were purchased from Gibco, USA. Human hepatic L02 cells were obtained from the Chinese Typical Culture Preservation Center (No: 3131C0001002000006). Cell Counting kit-8 (CCK-8) was purchased from Tongren Institute of Chemistry, Japan. PBS was purchased from Beijing Solarbio Technology Co., Ltd. Acetaminophen (China Food and Drug Regulatory Research Institute, China) was chosen as the positive control for cell experiments. CSG, trans-stilbene glycoside (TSG), emodin, physcion, EG, catechin, and gallic acid were purchased from Chengdu Preferred Biotechnology Co., Ltd., China. Moreover, the purities of all the standards were not <98%. Acetonitrile and methanol of ultra-high-performance liquid chromatography (UHPLC) grade (Sigma, USA) were used for the mobile phase of chromatographic analysis. Deionized water was purified by a Millipore’s ultrapure water system (Millipore, Bedford, USA).

**Standards and sample preparation**

1.0 kg of black soybean were extracted twice with moderate amounts of distilled water at 100°C (4 h per extraction) and then the combined extraction was condensed to 2.5 kg. After infiltrated with the black soybean liquid for 8 h, raw PM was steaming under high pressure for 6 h and drying without sun exposure in the modern processing group. In the NSSD groups, raw PM was infiltrated with the black soybean liquid for 8 h, steamed under high pressure for 6 h, and then placed outdoors for 24 h until completely dried. This is one time for steaming and sun drying. For the preparation with different processing times, repeat the procedure of steaming and sun drying until the ninth time.

Crude and different processed products of PM were crushed and sieved through a 40-mesh sieve (0.425 mm), then extracted with 50% ethanol under ultrasonic for 30 min, respectively. The extraction method has been proved that PM could be extracted completely and cause severe hepatotoxicity in a study. After filtration, distillation under reduced pressure, and vacuum drying, the dried extraction of PM was obtained and the yield was calculated. All extracts were dissolved in 50% methanol under ultrasonic and filtered through the 0.22 μm filter membrane before the UHPLC fingerprinting analysis.

Sample solution was prepared as follows: gallic acid, catechin, CSG, TSG, EG, physcion, and emodin were dissolved in 50% methanol (UHPLC grade) and filtered through the 0.22 μm filter membrane for the UHPLC fingerprinting analysis, with the final concentrations as follow: 50.100 μg/mL for gallic acid, 50.000 μg/mL for catechin, 50.900 μg/mL for CSG, 51.300 μg/mL for TSG, 48.800 μg/mL for EG, 53.100 μg/mL for physcion, and 49.200 μg/mL for emodin.

**Ultra-high-performance liquid chromatography conditions**

All samples were analyzed by the Agilent Technology 1290 Infinity UHPLC. The separation was performed on an Agilent 300SB-C18 column (2.1 mm × 100 mm, 1.8 μm) at 30°C. The mobile phase is water (A) and acetonitrile (B), and a gradient elution was set as follows: 5%–32% (v/v) B at 0–5 min, 32%–55% (v/v) B at 5–6 min, 55%–88% (v/v) B at 6–12 min, and 88%–90% (v/v) B at 12–15 min. The flow rate was 0.3 mL/min with a sample injection volume of 0.5 μL, and the detection wavelength was set at 280 nm.

**Method validation**

Sample solution of S1 was chosen randomly to test the feasibility and suitability of the experimental method and condition. The method precision was assessed by six successive injections of one sample solution, while repeat ability was estimated by six replicates of a sample from the same origin. For the storage stability test, replicate injections of one sample solution, which was stored in a volumetric flask at room temperature, were analyzed in a day (0, 2, 4, 6, 8, 12, and 24 h).

**Chemical fingerprint analysis**

Each sample solution was analyzed in triplicate to get the fingerprints with the optimized HPLC condition, respectively, and then chemical fingerprints were analyzed by the Similarity Evaluation System for Chromatographic Fingerprint of Chinese Medicine (Version 2004A). With the chromatogram of crude PM as the reference, and the window width was limited to 0.1 min. Then, the reference atlas was formed, and the
similarities between the reference atlas and the chromatogram of different extracts were calculated by this software.

**Peak identification**

The pure reference standard solutions were injected into the Agilent 1290 UPLC-6550 Q-TOF MS/MS (Agilent Technologies, USA) system for qualitative analysis and the retention time of reference standard was recorded. The mobile phase and the condition are same as 2.3. Electrospray capillary voltage was 3500 V in negative ionization mode. The temperature of dry gas was 200°C, and the flow rate of dry gas was 14 L/min; nebulizer pressure was set to 35 Psig. The temperature of sheath gas was 350°C, and the flow rate of sheath gas was 11 L/min; nozzle voltage was 1000 V; MS data were collected from m/z 100 to 1700 in the full scan mode. Some compounds were identified including gallic acid, catechin, CSG, TSG, EG, physcion, and emodin.

**Cell culture and cell viability assay**

Human hepatic cell line L02 was cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in humidified air with 5% CO₂ incubator. Raw and different processed PM decoction pieces were, respectively, extracted twice with 8 volumes of 50% (V/V) ethanol by cold soak for 48 h. The collected extraction of each group was combined and condensed to the corresponding concentration for later use. L02 cells were seeded at the density of 7 × 10⁵ cells/ml into a 96-well plate, 100 μL each well. After 24 h, we removed the supernatant and added into 40 mg/mL of extraction (calculated by raw PM). The acetaminophen solution (1.7 mg/mL) was used as the positive control, and six repeating pipettes were set in each group. After incubation for 24 h, the supernatant was removed, and then the CCK8 assay was employed to determine the viability of L02 cells. After incubation with the CCK-8 solution for 30min, the absorbance (A) of sample was determined at 450 nm. The absorbance of acetaminophen solution, experimental samples, and blank control groups was As, At, and Actr, respectively. Calculate the inhibition rate following the formula:

\[
\text{Inhibition rate} = (1 - \frac{[At - Actr]}{[As - Actr]}) \times 100\%
\]

**Spectrum-toxicity correlation**

Principal component analysis and cluster analysis

The original data of peak area of 18 common peaks \((X1 \sim X18)\) in UHPLC fingerprints were normalized, and the average of each group was calculated. Then, the dataset and toxicity data were imported into the SPSS 13.0 software, International Business Machines Corporation company, America, and unsupervised principal component analysis (PCA) was performed. The hierarchical clustering analysis of samples was processed to primarily show the relationship between the data matrices and screen potential hepatoxic compounds.

Simple correlation analysis

In order to independently investigate the correlation between each component and hepatoxicity without the other component variables, the simple correlation analysis was performed using SPSS 13.0 software. The cytotoxicity of PM extracts in groups was the dependent variable \((Y)\). The peak area of major components of PM was the independent variable \((Xi \sim X7)\), which were gallic acid, emodin, physcion, catechin, TSG, CSG, and EG, respectively. Due to \(X1, X2, \text{and} \ X5\) satisfied the normal distribution, the Pearson’s simple correlation was conducted, whereas \(X3, X4, X6, \text{and} \ X7\) were skew distribution, thus the Spearman’s simple correlation analysis was conducted. According to the correlation coefficient \(r > 0.95\) and \(P < 0.05\), the compositions relevant to hepatoxicity were screened.

**Multiple regression analysis**

The collinear diagnosis was conducted, and the conditional number was >30, which indicated that the independent variable was of severe collinearity. Therefore, the PCA was further employed to investigate the correlation between toxicity and compounds. According to the eigenvalues and contribution rates in the regression matrix, we extracted principal components among seven components, \(Z1 \text{and} \ Z2\). The cumulative contribution rate was 0.9556 (>0.85), and the other principal components were eliminated. The normalized formula \(Ai = \left(\frac{Xi - \bar{X}}{SD}\right)\) is (SD is the sample standard deviation). \(Z1\) and \(Z2\) were substituted into the regression model formula, and then only the \(Z1\) fitted it well. The expression of \(Z1\) was brought into the regression model, and the linear regression model of the dependent variable \((Y)\) to the standardized independent variables \((A1 \sim A7)\) was obtained. The values of \(A1 \sim A7\) were converted into the original independent variable \((X1 \sim X7)\) by the standardized formula, then the linear regression equation was obtained.

**Results and Discussion**

Method validation for ultra-high-performance liquid chromatography fingerprints

Method validation for UHPLC fingerprint results showed that the relative SD for method precision and reproducibility, alone with storage stability of sample solutions within 24 h appeared <3% both for relative retention time and average peak area of common peaks. It meant that the established fingerprint was satisfied.

**Chemical fingerprint profile and cytotoxicity**

L02 cell line, widely used in hepatotoxicity research, could maintain characteristics and ultrastructure of normal liver cells after continuous passage and express many specific liver cell functions. A total of 18 common chromatographic peaks in all groups were detected by the UHPLC system. As shown in Table 1 and Figure 1, there were differences in chemical fingerprints and hepatoxicity between crude PM and processed products during the NSSD method. With the repeated times of steaming and sun drying, the chemical fingerprint similarity with the raw declined from 0.985 to 0.113, and the L02 cell inhibition rate obviously decreased as well. The results suggested that the toxicity attenuation by the nine-time steaming and sun drying method...
Toxicity attenuation by the nine-time steaming and sun drying method

Table 1: Chemical fingerprint similarity and cell inhibition rate of Polygoni Multiflori extracts

<table>
<thead>
<tr>
<th>Number</th>
<th>Analytes</th>
<th>Chemical fingerprint similarity</th>
<th>Cell inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Crude PM</td>
<td>1.000</td>
<td>93.43 ± 2.54</td>
</tr>
<tr>
<td>S2</td>
<td>Steaming with black soybean liquid</td>
<td>0.993</td>
<td>76.06 ± 1.98</td>
</tr>
<tr>
<td>S3</td>
<td>One time of NSSD</td>
<td>0.985</td>
<td>71.73 ± 1.37</td>
</tr>
<tr>
<td>S4</td>
<td>Three times of NSSD</td>
<td>0.900</td>
<td>62.24 ± 3.24</td>
</tr>
<tr>
<td>S5</td>
<td>Five times of NSSD</td>
<td>0.766</td>
<td>41.21 ± 1.76</td>
</tr>
<tr>
<td>S6</td>
<td>Seven times of NSSD</td>
<td>0.501</td>
<td>29.09 ± 1.67</td>
</tr>
<tr>
<td>S7</td>
<td>Nine times of NSSD</td>
<td>0.113</td>
<td>38.37 ± 2.07</td>
</tr>
</tbody>
</table>

PM: Polygoni multiflori, NSSD: Nine-time steaming and sun drying

Spectrum-toxicity correlation analysis

The above experimental results revealed that crude and processed PM had significant differences in hepatotoxicity and chemical compositions. In order to further clarify the correlation between chemical components and toxicity, multiple statistical methods were conducted to screen the toxicity-related components.

Assignment of major hepatotoxicity-related components

The correlation matrix was applied to visualize the spectrum-toxicity correlation in Figure 3. The 18 common compounds were shown and illustrated the closeness relationships with hepatotoxicity. Compounds were clearly divided into three main clusters: positive correlation, negative, and no significant correlation with hepatotoxicity. Due to the decreased contents of hepatotoxic components, the hepatotoxicity of PM extracts attenuated with the times of processing. Thus, the hepatotoxic components probably were in the cluster of positive correlation with hepatotoxicity, that is, among seven compounds (No. 3, 5, 6, 8, 9, 11, and 13). In additiona, these compounds had stronger correlation with each other.

Combined with the PCA analysis, seven compounds were screened and identified, which changed significantly during the processing and had positive correlation with hepatotoxicity. They were gallic acid (No. 3), catechin (No. 5), CSG (No. 6), TSG (No. 8), EG (No. 9), physcion (No. 11), and emodin (No. 13).[25,26]

Multiple statistical analysis

As shown in Table 2, the simple correlation analysis suggested that CSG, EG, and physcion were the most relevant to the hepatotoxicity of PM, with both the correlation coefficient \( r > 0.95 \) and \( P < 0.05 \) as indicators.

Although the spectrum-toxicity correlation is a good approach to analyze the potential toxic compounds from complex compositions of herbs, it is dependent on the statistical analysis to some extent. Thus, another statistical method, the multiple regression analysis, was employed to comprehensively investigate it. The regression equation was obtained follow as: \[ Y = 1.40 - 3.06 \times 10^{-7} X_1 + 3.24 \times 10^{-8} X_2 + 1.09 \times 10^{-6} X_3 + 1.27 \times 10^{-6} X_4 + 9.85 \times 10^{-7} X_5 + 3.47 \times 10^{-7} X_6 + 5.66 \times 10^{-8} X_7. \] (\( R^2 = 0.7366, P = 0.0009 < 0.01 \)). The results were of statistically significant in the regression model. It

chemical constituents of PM varied during the NSSD processing, which may influence efficacy and toxicity of PM. After five repeated steaming and sun drying, the cell inhibition rate of PM extractions was below 50%, whereas seven repeated processing showed a better effect on toxicity attenuation. Furthermore, the modern processed group which PM was steamed with black soybean liquid for 6 h without sun drying, exhibited less effect of toxicity attenuation than the NSSD groups.

Analysis of common compositions

Through the PCA, the score scatter plot reflecting the degree of dispersion between groups was rendered. As shown in Figure 2a, all data were distributed within the 95% confidence interval (elliptical region) without any outlier. The processed groups deviated the raw group to varying degrees and extended along t1. Although both the modern processed group (S2) and the NSSD groups (S3–S7) distinguished from the raw PM, the NSSD groups varied more enormously. The above results suggested that the NSSD processing had a great influence on the constitute changes of PM. Interestingly, there were differences in chemical components between the NSSD groups and modern processed group. Combined with the Figure 2b, we found that the 18 common components showed different variation trends during the NSSD processing. Some components had significant differences in content including No. 2, 3, 4, 5, 6, 8, 9, 10, 11, 13.14, and 15.

Figure 1: Fingerprint chromatogram of crude and processed PM. 3: gallic acid; 5: catechin; 6: cis-stilbene glycoside; 8: trans-stilbene glycosides; 9: emodin-8-O-β-D-glucoside; 11: physcion; and 13: emodin.

Figure 2: Score scatter plot of principal component analysis. a: raw group; b: modern processed group; c: NSSD group. Each compound was coated by number and colored as channel 1 (red), channel 2 (blue), channel 3 (green), channel 4 (yellow), channel 5 (orange) and channel 6 (light yellow). A6–A11 were the clusters of positive correlation with hepatotoxicity, that is, among seven compounds (No. 3, 5, 6, 8, 9, 11, and 13). A7–A13 were the clusters of negative correlation with hepatotoxicity. A1–A5 were the cluster of no significant correlation with hepatotoxicity.
could be seen, $X_3$, $X_4$, and $X_6$ had larger parameter values, which indicated that physcion, catechin, and CSG had greater contribution to the hepatotoxicity of PM.

Through simple correlation analysis and multiple linear regression analysis, total four compounds of potential hepatotoxic were screened. Among them, CSG and EG were major compounds related to the susceptibility of PM-induced hepatotoxicity which was confirmed by our previous studies. Then, the content changes of four components during the NSSD processing were investigated.

**The content alteration trends of hepatotoxicity-related components**

The appearance, compositions, and toxicity of PM underwent extremely complex changes during the NSSD processing. The PM products shrunk and lost volume which color gradually changed from tan to dark brown. With the repeated times of steaming and sun drying, the texture of PM products first became solid and then loose; meanwhile, they became brittle with sweet fragrance. The internal color of PM decoction pieces deepened with the processing. After five-times steaming and drying, both the inside and outside were dark brown, and the yield of PM products remained stable within the range of 73%–84%. It was difficult to identify the processing times of PM only by the changes of appearance.

Compared with the crude group, the contents of four hepatotoxic-related components decreased during the NSSD processing, which was consistent with the alteration trend of hepatotoxicity. The relative change rate of concentration in

<table>
<thead>
<tr>
<th>Number</th>
<th>$Y$ (%)</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>$X_4$</th>
<th>$X_5$</th>
<th>$X_6$</th>
<th>$X_7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>93.43</td>
<td>792,620</td>
<td>35,846,072</td>
<td>872,353</td>
<td>804,133</td>
<td>12,277,972</td>
<td>294,739</td>
<td>18,491,905</td>
</tr>
<tr>
<td>S2</td>
<td>76.06</td>
<td>3,169,652</td>
<td>28,309,848</td>
<td>668,905</td>
<td>53,480</td>
<td>7,217,887</td>
<td>231,176</td>
<td>12,548,908</td>
</tr>
<tr>
<td>S3</td>
<td>71.73</td>
<td>1,987,483</td>
<td>16,696,331</td>
<td>67,563</td>
<td>21,500</td>
<td>4,164,557</td>
<td>47,641</td>
<td>3,470,492</td>
</tr>
<tr>
<td>S4</td>
<td>62.24</td>
<td>2,217,287</td>
<td>17,748,932</td>
<td>37,325</td>
<td>0</td>
<td>2,704,514</td>
<td>26,957</td>
<td>1,635,620</td>
</tr>
<tr>
<td>S5</td>
<td>41.21</td>
<td>3,116,112</td>
<td>19,725,487</td>
<td>20,294</td>
<td>0</td>
<td>2,007,264</td>
<td>28,674</td>
<td>72764.2</td>
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<tr>
<td>S6</td>
<td>29.09</td>
<td>2,658,882</td>
<td>16,744,409</td>
<td>9,977</td>
<td>0</td>
<td>1,035,081</td>
<td>22,240</td>
<td>17488.22</td>
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<tr>
<td>S7</td>
<td>38.37</td>
<td>2,139,057</td>
<td>198,408</td>
<td>0</td>
<td>0</td>
<td>43,613</td>
<td>9,641</td>
<td>0.964</td>
</tr>
</tbody>
</table>

$r$ | 1.000 | −0.555 | 0.738 | 0.964 | 0.906 | 0.910 | 0.964 |

$P$ | 0.196 | 0.058 | 0.000 | 0.005 | 0.004 | 0.000 | 0.000 |

all groups was calculated, which showed that four chemical compositions were steady after five times of the NSSD. It indicated that the nine times of NSSD method recorded in ancient books probably meant the repeated steaming and sun drying for multiple times, rather than exact nine times.\[15]\n
As shown in Figure 4, EG, CSG, and catechin were almost eliminated through repeated steaming and sun drying. Through repeated steaming and sun drying, EG, CSG, and catechin were almost eliminated. During the processing, the catechin was transformed into condensation product at high temperature. The content of physcion was constantly fluctuant, probably because there were different transformation rates between free and bound anthraquinones. The contents of catechin and physcion declined rapidly after the one time of NSSD and remained stable until the nine times of NSSD. Based on the alteration trend, they had good potential to indicate whether PM products processed by steaming and sun drying.

**Conclusions**

The spectrum-toxicity correlation study provides a good approach to analyze the potential toxicity-related compounds from complex compositions of herbs.\[27,28\] It is significant to employ multiple and comprehensive statistical methods to reveal the variation trends in depth. In this study, we established the chemical fingerprints of crude and processed PM and investigated the spectrum-toxicity correlation by UHPLC and comprehensive statistical analysis. The results suggested that the NSSD method posed a great influence of toxicity attenuation to PM, which was superior to the method by steaming with black soybean liquid. We screened four major hepatotoxicity-related components. Among them, we confirmed the susceptibility-related compounds of PM-induced liver injury as EG and CSG and found other two compounds, catechin and physcion, that could be employed to identify whether the PM products underwent steaming and sun drying.

In conclusion, this study primarily revealed the scientific connotation of NSSD processing of PM which could provide data support for hepatotoxicity research and might also deliver a feasible strategy for evaluating the toxicity attenuation and ensuring the safety use of PM in clinic.

**Acknowledgments**

This work was funded by the National Natural Science Foundation of China (Grant No. 81630100) and the National Industry Program of China (Grant No. 201507002).

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

**References**


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