Determining the effective compounds of *Salvia miltiorrhiza* through chromatographic fingerprinting coupled with chemometrics

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ABSTRACT

Herbs are rich in the active ingredients of drugs for preventing or treating various disorders. However, conventional bioactivity-guided separation is time and labor-intensive and neglects the additive effect of multiple components. These problems hinder the development of new medicines from natural products. This study established a chemometric analysis method that integrates processes based on the spectrum-effect relationship for the rapid identification of the primary active components of a plant. The high-performance liquid chromatography (HPLC) fingerprints of 171 *Salvia miltiorrhiza* extracts (SMEs) with varied constituent profiles were analyzed. Chemometric analysis was performed to establish an HPLC fingerprint–bioactivity relationship to explore the components of SMEs that contribute to the antioxidant activity and cytotoxicity effect, respectively. The results indicated that the developed strategy can be used to identify components largely contributing to particular bioactivities and re-evaluate the efficacy of previously neglected components. The present study identified not only the primary active components of *S. miltiorrhiza* but also the optimal ratios of constituents, validating the method for use in the future investigation and development of herbal medicines.

**Keywords:** Chemometric, herb, antioxidant, cytotoxicity, fingerprint, *Salvia miltiorrhiza*.

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**Abbreviations:** TCM, traditional Chinese medicine; SME, *Salvia miltiorrhiza* extract; HPLC, high-performance liquid chromatography; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; DPPH, 2,2-diphenyl-1-picyrylhydrazyl; HCA, hierarchical clustering analysis; PCA, principal component analysis; MLR, multiple linear regression; TAC, total antioxidant capacity.

INTRODUCTION

Natural compounds isolated from medicinal herbs are vital for drug discovery (Atanasov et al. 2021). The root of *Salvia miltiorrhiza* Bunge (Lamiaceae), also known as *danshen* in Chinese, has been used in traditional Chinese medicine (TCM) for millennia to treat and prevent various diseases (Yin et al., 2021; Han et al., 2021), such as coronary heart diseases, hyperlipidemia, and cerebrovascular disease (Wang et al., 2017). Pharmacological experiments have indicated that *S. miltiorrhiza* extracts (SMEs) exhibit numerous types of biological activity, including cardioprotective, neuroprotective, anti-inflammatory, and antitumor (Ho and Chang, 2015; Jiang et al., 2019). The primary phytochemical constituents of SMEs can be categorized
into two groups: water-soluble phenolic acids (e.g., salvianolic acid) and lipophilic diterpene quinones (Chen et al., 2014). Tanshinone I, tanshinone IIA, dihydrotanshinone, and cryptotanshinone are the primary lipophilic active constituents of S. miltiorrhiza, and numerous in vitro and in vivo studies have revealed antitumor activities of these primary tanshinones and their underlying mechanisms (Ho and Chang, 2015).

Determining which components of S. miltiorrhiza can be used in pharmacological treatments for various diseases is essential and difficult. Currently, conventional bioactivity-guided separation is limited because it is time and labor-intensive and it neglects the synergistic effect of multiple components. We previously used bioassay-guided fractionation to isolate and identify the active constituents of SMEs that exert cytotoxic and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)—sensitizing effects against an array of human cancer cell lines (Chang et al., 2013). Potential drug targets were investigated based on established knowledge of the chemical composition of S. miltiorrhiza. Therefore, some chemical constituents not yet considered useful in herbal medicine research might have been neglected. In addition, the phytochemical constituents of S. miltiorrhiza may vary with genotype, harvesting period (Liu et al., 2016), the drying process, and environmental factors (Peng et al., 2014). Accordingly, the proportion of these components in herbs differ and thus disparately affect the herbs’ pharmacological activities (Ma et al., 2007; Zhong et al., 2009).

Chromatographic fingerprint analysis is a useful method of identifying the components (Hamburger, 2019; Ba et al., 2021) food authentication activities (Esteki et al., 2020), geographical origin (Wang et al., 2020; Pan et al., 2020), and evaluating the quality of a TCM (Li et al., 2020). The HPLC fingerprint analysis is an established method of assessing herbal medicines (Long et al., 2021). However, through chemometric methods, such as multivariate analysis, regression analysis, and artificial neural network analysis, the relationship between fingerprints and bioactivity can be established, enabling the investigation of the particular components that contribute to certain bioactivities (Kong et al., 2017; Shi et al., 2018; Razmowski-Naumovski et al., 2020). The findings provide a general approach for examining the spectrum–effect relationships in the HPLC fingerprints as well as the antioxidant and cytotoxicity activities of medicinal plants.

MATERIALS AND METHODS

Experimental design

This study established a chemometric analysis method that assesses spectrum–effect relationships for the rapid determination of the primary active components of S. miltiorrhiza. Figure 1 presents the four primary components of this strategy: (1) phytochemical variability analysis, which involves the preparation of 171 SMEs with various constituent profiles; (2) spectrum–effect relationship analysis of the HPLC fingerprints and bioactivities of the SME samples; (3) chemometric analysis, which includes hierarchical clustering analysis (HCA) and principal component analysis (PCA); and (4) multiple linear regression (MLR) analysis, which reveals the components primarily contributing to the bioactivities of the SMEs.

Preparation of SMEs with constituent variability

Salvia miltiorrhiza was provided by the Biotechnology Division of the Taiwan Agricultural Research Institute. Cultivars were propagated vegetatively through cuttings, grafting, and even tissue culture. The sample number of each S. miltiorrhiza line was determined by the condition of the health status and integrity of the plant while harvesting. In this study, the lowest sample number was 3 for the 4N2-12 line due to serious disease problems, and the average sample number for the rest of the S. miltiorrhiza lines was around 7.6. In total, 171 extract samples of S. miltiorrhiza of 23 lines were collected in 4 different harvest periods in the Taichung area of Taiwan. The SMEs were prepared as previously described (Chang et al., 2013). The plant roots were subjected to conventional preliminary treatments, such as cleaning, cutting, and dry heating at 50°C. The dried plants were ground using an electric mill, sieved (6-mm filter), and stored at 4°C. Moreover, a 0.2 ± 0.001 g dry slice of S. miltiorrhiza was accurately weighed, mixed with 20 ml of 100% methanol for 30 min, and sonicated (100 W) at 37 to 40°C for another 30 min. Each SME was centrifuged for 5 min at 4000 rpm, and the supernatant was then transferred into a volumetric flask. This extraction process was repeated three times, and all supernatants were combined with the sediment and maintained at 4°C overnight. All extracts were centrifuged for 5 min at 12000 rpm and filtered through a 0.45 μm polyvinylidene fluoride syringe filter. The filtrates were dried under reduced pressure and resuspended in 100% methanol to form a 20 mg/ml sample solution. This solution was then stored in dark at 4°C for future usage.

Activity profiling using HPLC analysis

HPLC fingerprinting is a simple, sensitive, and accurate method to determine the chemical composition of herb samples (Yang et al., 2011). A Waters alliance 2695 HPLC system equipped with a Waters 2489 UV/Visible (UV/Vis) detector was used to analyze the composition of 171 SMEs. Chromatographic separations were performed on a X-Bridge™ Shield RP 18 column (4.6 × 250 mm, 5 μm; Waters). The column temperature was set at 35°C, and eluted compounds were detected by the UV/Vis detector at 254 nm. The gradient condition was as follows: MeOH:0.02% H3PO4:ACN = 0.75:25 → 5:60:35 (20 min) → 10:45:45 (30 min) → 27:16:57 (60 min). The flow rate was set at 1.0 ml/min; the injection volume was 20 μl. All solvents were of HPLC quality, and all chemicals were of analytical grade (>98%). The water was of Milli-Q quality. The identification of the phenolic compounds of the sample was based on a comparison between their retention times (T R’s) with those of commercial standards. Commercial standards for salvianolic acid B, dihydrotanshinone, cryptotanshinone, tanshinone Ia, and tanshinone IIA were purchased from ChromaDex™, Inc. (Irvine, USA).

Antioxidant activity

The antioxidant capacities of 171 SMEs were established by using two antioxidant assays; the reduction of potassium ferricyanide (Adjiman and Asare, 2015) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Cheng et al., 2006).
Figure 1. Experimental design. The four primary steps of this strategy are as follows: (1) Phytochemical variability: the preparation of 171 *Salvia miltiorrhiza* extracts (SMEs) with constituent variability; (2) Spectrum–effect relationship: an HPLC fingerprint analysis for characterizing chemical constituents of SMEs. The identification of antioxidant activities and cytotoxicity effect of SME samples; (3) Chemometric analysis: hierarchical clustering analysis (HCA), principal component analysis (PCA); and (4) Validation and prediction: multiple linear regression (MLR) analysis showed that the putative components primarily contributed to bioactivities.

**Reduction of potassium ferricyanide using L-ascorbic acid**

The substances reacting with potassium ferricyanide \([K_3Fe(CN)_6]\) to form potassium ferrocyanide \([K_4Fe(CN)_6]\) have a reduction potential. Therefore, 150 μL of each sample was mixed with 150 μL of 200 mM sodium phosphate buffer (pH 6.6) and 150 μL of 1% K₃Fe(CN)₆. After incubation at 50 °C for 20 min, the mixture was rapidly cooled on ice, and 150 μL of 10% trichloroacetic acid was added. The mixture was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution was transferred at 100 μL/well to a 96-well plate, followed by the addition of 100 μL of distilled water and 20 μL of 0.1% FeCl₃ solution. The plate was then incubated for 10 min at room temperature in a dark place before absorbance was measured at 700 nm. Ascorbic acid (0.2 mg/ml) was used as a positive control. The antioxidant capacity was calculated as \((A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100\%\). All experiments were repeated at least three times for triplicated samples in each experiment.

**DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging activity**

Antioxidant activity was obtained using the DPPH radical scavenging method as previously described. In brief, 100 mM methanolic DPPH was prepared. An aliquot (500 μL) of each sample (with appropriate enrichment or dilution if...
required) was added to 500 μL of a methanolic DPPH solution for initiating the reaction. Discolorations were measured at 528 nm after a reaction for 30 min at room temperature in the dark. The radical scavenging activity was calculated as a percentage of DPPH discoloration by using the following equation: DPPH radical scavenging percentage = \( [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100\% \). All experiments were repeated at least three times with triplicated samples in each experiment.

**Cytotoxicity effect**

Human alveolar lung epithelial carcinoma (A549) and human ovarian clear cell carcinoma (TOV-21G) were used in this study. A549 cells were developed in a medium composed of 90% RPMI 1640 and 10% fetal bovine serum supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin. TOV-21G cells were grown in a 1:1 mixture of MCDB 105 and medium 199 with 15% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were allowed to grow at 37°C in a humidified 5% CO₂ atmosphere. Cells were subjected to a 48-h treatment with 20 μg/mL of extracts. Cytotoxicity effect was assayed with [3-(4,5-diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] inner salt (MTS) in accordance with the established protocol (Chang et al., 2015). All experiments were repeated at least three times with triplicated samples in each experiment.

**Hierarchical clustering analysis (HCA)**

To uncover the similarity relationship of the 23 S. miltiorrhiza lines, the HCA was performed. In total, 171 observations of HPLC fingerprints were analyzed using the HCA on the peak area of 17 common peaks with Heml (Heatmap Illustrator, version 1.0.1). The HCA was conducted by analyzing the 17 peaks of 23 S. miltiorrhiza lines, and each line had a different number of observations, hence it should be a 17 (peaks) × 23 (plant lines) matrix. The Euclidean distance was used as a similarity metric, the 23 S. miltiorrhiza lines were classified into clustering groups based on the average linkage method. The results showed a two-way clustering matrix with dendrograms appended to its margins.

**Principal component analysis (PCA)**

The PCA is a dimension-reduction statistical method, which minimizes data but retains crucial information. In this study, the bioactivities including K₃Fe(CN)₆ reduction assay, free radical-scavenging, cytotoxicity A549, and TOV-21G were used as variables to cluster 23 lines of SME samples and each variable has 171 observations. In the PCA, the singular value decomposition of the data matrix was performed, this matrix values for the four variables of 23 S. miltiorrhiza lines by using pca3d package (version 0.10.2) in R software (3.6.0).

**MLR analysis**

MLR is a statistical technique that uses several independent variables to predict the outcome of a dependent variable. Each value of the independent variables \((X_1, \ldots, X_k)\) is related to a dependent-variable value \((Y)\). The MLR equation is typically expressed as follows:

\[
Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_k X_k
\]

Where \(Y\) is the dependent variable, \(X_1\) through \(X_k\) are \(k\) distinct independent variables, \(\beta_0\) is the value of \(Y\) when all of the independent variables \((X_1\) through \(X_k)\) are zero, and \(\beta_1\) through \(\beta_k\) are the estimated regression coefficients. Each regression coefficient represents the change in \(Y\) relative to a one-unit change in the respective independent variable. In this study, MLR analysis was performed to determine the relationship between the bioactivities and fingerprints of the 171 SME samples. Data on each bioactivity of the 171 samples were used as dependent variables \((Y_1 - Y_4)\), and the peak areas of 17 compounds \((A-Q)\) Figure 2 were used as independent variables \((X_1 - X_{17})\) to fit an MLR model in SAS Enterprise Guide version 7.1 (SAS Institute Inc, Cary, NC). To further explore the active compounds of \(S. miltiorrhiza\) that significantly contribute to each bioactivity, stepwise variable selection with \(p < 0.05\) was introduced to the MLR model. The adjusted-\(R^2\) (Adj.-\(R^2\)) for the MLR model was calculated as follows:

\[
\text{adjusted } R^2 = 1 - \frac{SS_{\text{res}}}{SS_{\text{total}}}
\]

where \(SS_{\text{res}}\) and \(SS_{\text{tot}}\) represent the residue sum of squares and the total sum of squares, \(n\) is the sample size and \(p\) is the number of independent variables.

**RESULTS**

**HPLC fingerprints of SMEs**

In total, 171 SME samples were analyzed, and approximately 17 peaks were found in each sample. The process of standardization included the selection of common peaks in chromatograms and the normalization of \(T_R\) values of all common peaks. Appropriately separated and large-area peaks were selected. Although quantifying substances that do not belong to common peaks may be desirable, it is not always achievable because sometimes identifying such substances is difficult. Figure 2 illustrates a representative standard fingerprint; the characteristic peaks are labeled based on their elution order (peaks 1–17). Among the common peaks, six known compounds were identified by matching their \(T_R\) to that of their respective standard compounds. The retention time \((T_R)\) for the standard solutions of salvianolic acid B, dihydrotanshinone, cryptotanshinone, tanshinone I, and tanshinone IIA is 11.51, 35.62, 40.71, 43.55, and 48.76 min, respectively.

**Hierarchical clustering analysis (HCA)**

According to chromatograms obtained using all samples, 17 common peaks were selected and calculated their relative retention times and relative peak areas were then calculated. Figure 3 shows the results of a hierarchical cluster analysis. A matrix was applied for HCA by using SPSS software, which comprised the number of samples and the relative peak area of 17 constituents. A heatmap was devised to display the visually identify patterns of hierarchical clustering trees. A dendrogram was acquired
Figure 2. HPLC chromatograms of SMEs under 254 nm. (a) Compounds at peaks A–Q were used for multivariate analysis. Compounds at peaks D, J, M, N, and P were salvianolic acid B (RT = 11.51 min), dihydrotanshinone (RT = 35.62 min), cryptotanshinone (RT = 40.71 min), tanshinone I (RT = 43.55 min), and tanshinone IIA (RT = 48.76 min), respectively. (b) Fingerprint of SMEs with the retention time of compounds at peaks A–Q in the HPLC analysis.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>( T_R ) (min)</th>
<th>Standard compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.93</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>8.89</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>9.63</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>11.51</td>
<td>Salvianolic acid B</td>
</tr>
<tr>
<td>E</td>
<td>3.29</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>13.91</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>14.69</td>
<td>-</td>
</tr>
<tr>
<td>H</td>
<td>20.65</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>30.16</td>
<td>-</td>
</tr>
<tr>
<td>J</td>
<td>35.62</td>
<td>Dihydrotanshinone</td>
</tr>
<tr>
<td>K</td>
<td>36.67</td>
<td>-</td>
</tr>
<tr>
<td>L</td>
<td>37.71</td>
<td>-</td>
</tr>
<tr>
<td>M</td>
<td>40.71</td>
<td>Cryptotanshinone</td>
</tr>
<tr>
<td>N</td>
<td>43.55</td>
<td>Tanshinone I</td>
</tr>
<tr>
<td>O</td>
<td>45.37</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>48.76</td>
<td>Tanshinone IIA</td>
</tr>
<tr>
<td>Q</td>
<td>49.71</td>
<td>-</td>
</tr>
</tbody>
</table>

Using an average linkage between groups and the cosine method, the relative peak areas of these common peaks were calculated, thus forming a 17 (peaks) \( \times \) 23 (plant lines) matrix before HCA. The matrix was calculated using SPSS, thus generating a dendrogram, which indicated similarities between the samples. The dendrogram showed that the 23 plant lines were divided into four primary clusters: I, II, III and IV. Because samples in the same cluster possessed similar qualities and characteristics. The 23 lines of *S. miltiorrhiza* were grouped into four primary clusters: 3-7_S, 4N2-12, 4N2-7, and 4N3-7 lines were categorized into cluster I; 99P01, 99P02, I_R, I-S, and MS04_S lines into cluster II; H_R, PS14, H_S, P_S, PS12, MS04_R, P_R, and PS04 lines into cluster III; and MS03_R, MS03-1_R, PS15_R, MS06, and IS18 lines into cluster IV. Compared with
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Figure 3. HCA of 23 lines of SME samples was performed using Heatmap Illustrator (version 1.0.1). Euclidean distance was used as a similarity metric. Clustering groups were amalgamated based on the average linkage method.

S. miltiorrhiza lines in separated clusters, S. miltiorrhiza lines in the same cluster exhibited short distances. Therefore, HPLC chemical fingerprints of the S. miltiorrhiza lines within the same clusters were more similar than those in different clusters.

Principal component analysis (PCA)

The antioxidant activity of S. miltiorrhiza was evaluated using a potassium ferricyanide ([K₃Fe(CN)₆]₃ reduction method and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The cytotoxic activity was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay in human alveolar lung epithelial carcinoma (A549), and human ovarian clear cell carcinoma (TOV-21G) cancer cells. A three-dimensional plot indicated that 23 S. miltiorrhiza lines were divided into four groups (Groups 1 to 4), and the first three principal components (PC1/PC2/PC3) contributed to 98.9% of the total variance based on the PCA using the results of K₃Fe(CN)₆ reductivity, free radical scavenging activity, cytotoxicity of A549, and TOV-21G as variables (Figure 4). The S. miltiorrhiza lines were categorized into distinct groups, which was consistent with the clustering results of HCA (Table 1).

MLR analysis

MLR is one of the intuitive statistical methods that had widely applied to discover chemical compounds with a significant effect on biological activity. After fitting a linear equation with the MLR model and performing a variable selection procedure, four satisfactory regression models with \( p < 0.001 \) were obtained, and predictor variables in each model were related to mean responses \( Y_1 \), \( Y_2 \), \( Y_3 \), and \( Y_4 \). In this study, antioxidant activities were determined using a K₃Fe(CN)₆ reduction method and DPPH radical scavenging activity. The first model indicated that compounds G, B, and E showed a positive impact, whereas compound M showed a negative impact on DPPH free radical scavenging (Table 2). Compounds D (salvianolic acid B), B, G, N (tanshinone I), and H showed a positive impact, whereas compound K showed
Figure 4. Three-dimensional plot of PCA. The first three principal components (PC1/PC2/PC3) contributed to 98.9% of the total variance. The 23 *S. miltiorrhiza* lines were divided into four groups. Group 1: yellow circles; Group 2: blue triangles; Group 3: indigo squares; and Group 4: yellow diamonds.

Table 1. Various origins and clustering results of 23 *S. miltiorrhiza* lines.

<table>
<thead>
<tr>
<th>No.</th>
<th>Line</th>
<th>Ploidy</th>
<th>HCA cluster</th>
<th>PCA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-7_S</td>
<td>Normal</td>
<td>I</td>
<td>Group 1</td>
</tr>
<tr>
<td>2</td>
<td>4N2-12</td>
<td>Chromosome doubling</td>
<td>I</td>
<td>Group 1</td>
</tr>
<tr>
<td>3</td>
<td>4N2-7</td>
<td>Chromosome doubling</td>
<td>I</td>
<td>Group 1</td>
</tr>
<tr>
<td>4</td>
<td>4N3-7</td>
<td>Chromosome doubling</td>
<td>I</td>
<td>Group 1</td>
</tr>
<tr>
<td>5</td>
<td>99P01</td>
<td>Normal</td>
<td>II</td>
<td>Group 2</td>
</tr>
<tr>
<td>6</td>
<td>99P02</td>
<td>Normal</td>
<td>II</td>
<td>Group 2</td>
</tr>
<tr>
<td>7</td>
<td>H_R</td>
<td>Normal</td>
<td>III</td>
<td>Group 3</td>
</tr>
<tr>
<td>8</td>
<td>H_S</td>
<td>Normal</td>
<td>III</td>
<td>Group 3</td>
</tr>
<tr>
<td>9</td>
<td>I_R</td>
<td>Normal</td>
<td>II</td>
<td>Group 2</td>
</tr>
<tr>
<td>10</td>
<td>I_S</td>
<td>Normal</td>
<td>II</td>
<td>Group 2</td>
</tr>
</tbody>
</table>
Table 1. Continues.

<table>
<thead>
<tr>
<th>MS03_ R</th>
<th>Normal</th>
<th>IV</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS18_ R</td>
<td>Normal</td>
<td>IV</td>
<td>Group 4</td>
</tr>
<tr>
<td>MS03-1</td>
<td>Normal</td>
<td>IV</td>
<td>Group 4</td>
</tr>
<tr>
<td>MS04_ R</td>
<td>Normal</td>
<td>III</td>
<td>Group 3</td>
</tr>
<tr>
<td>MS04_ S</td>
<td>Normal</td>
<td>II</td>
<td>Group 2</td>
</tr>
<tr>
<td>MS06_ R</td>
<td>Normal</td>
<td>IV</td>
<td>Group 4</td>
</tr>
<tr>
<td>P_ R</td>
<td>Normal</td>
<td>III</td>
<td>Group 3</td>
</tr>
<tr>
<td>PS04_ R</td>
<td>Normal</td>
<td>III</td>
<td>Group 3</td>
</tr>
<tr>
<td>PS08_ R</td>
<td>Normal</td>
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<td>Group 1</td>
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<tr>
<td>PS12_ R</td>
<td>Normal</td>
<td>III</td>
<td>Group 3</td>
</tr>
<tr>
<td>PS14_ R</td>
<td>Normal</td>
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<td>Group 3</td>
</tr>
<tr>
<td>PS15_ R</td>
<td>Normal</td>
<td>IV</td>
<td>Group 4</td>
</tr>
<tr>
<td>P_S</td>
<td>Normal</td>
<td>III</td>
<td>Group 3</td>
</tr>
</tbody>
</table>

Table 2. MLR models using the data of four bioactivities of the 171 extract samples as response variables

<table>
<thead>
<tr>
<th>Bioactivity</th>
<th>Regression model</th>
<th>Predictor variables</th>
<th>p</th>
<th>adj-R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH scavenging</td>
<td>( Y_1 = 0.68G + 0.24B + 0.14E - 0.13M )</td>
<td>B: +*** C: + D: + E: +*** G: - H: - J: - K: - M: - N: - P: -</td>
<td>&lt;0.001</td>
<td>0.652</td>
</tr>
<tr>
<td>Reduction of ( K_d[Fe(CN)_6] )</td>
<td>( Y_2 = 0.38D + 0.28B + 0.24G + 0.21N + 0.18H - 0.42K )</td>
<td>B: +*** C: +*** D: +*** E: +*** F: -*** G: +*** H: +*** J: +*** K: +*** M: +*** N: +***</td>
<td>&lt;0.001</td>
<td>0.696</td>
</tr>
<tr>
<td>Cytotoxicity effect (TOV-21G)</td>
<td>( Y_3 = 0.95P + 0.18B + 0.15C - 0.34D )</td>
<td>B: +*** C: + D: -*** E: -*** F: -*** G: -*** H: -*** J: -*** K: -*** M: -*** N: -***</td>
<td>&lt;0.001</td>
<td>0.758</td>
</tr>
<tr>
<td>Cytotoxicity effect (A549)</td>
<td>( Y_4 = 0.51K + 0.20P + 0.19J )</td>
<td>B: + C: + D: +*** E: -*** F: -*** G: -*** H: -*** J: -*** K: -*** M: -*** N: -***</td>
<td>&lt;0.001</td>
<td>0.741</td>
</tr>
</tbody>
</table>

D: salvianolic acid B, J: dihydrotanshinone, M: cryptotanshinone, N: tanshinone I, P: tanshinone IIA; +: Positive effect on the mean response of the model, −: Negative effect on the mean response of the model, The magnitude of predictor variables effect; *: \( p < 0.05 \), **: \( p < 0.01 \), ***: \( p < 0.001 \).

a negative impact on the antioxidant activity of iron chelators \( K_d[Fe(CN)_6] \) for the second model. Likewise, compounds P (tanshinone IIA), B, and C exhibited considerably positive effects, and compound D (salvianolic acid) showed a negative effect on the cytotoxicity of TOV-21G in the third model. However, the results of the fourth model revealed that compounds K, P (tanshinone IIA), and J (dihydrotanshinone) had a positive effect on the cytotoxicity of A549. The adjusted-\( R^2 \) (Adj-\( R^2 \)) of the four models were as follows: 0.652, 0.696, 0.758, and 0.741.

DISCUSSION

This study explored the spectrum–effect relationships between the HPLC fingerprints and bioactivity of \( S. miltiorrhiza \) through chemometric analysis to identify the primary compounds affecting its bioactivities. To this end, the HPLC fingerprints of 171 samples with constituent variability extracted from 23 \( S. miltiorrhiza \) lines harvested during four distinct periods were obtained (Figure 1). In PCA using four bioactivities as variables, the first three principal components (PC1, PC2, and PC3) contributed to 98.9% of the total variance (Figure 4). Table 2 presents the primary components. The MLR analysis revealed that the aforementioned components primarily contributed to SME bioactivities.

HCA enables the quantitative and objective analysis of HPLC fingerprints (Viaplana et al.,
In the heat map generated through HCA, the 23 S. miltiorrhiza lines were categorized into four clusters such that the lines in each cluster had similar profiles (Figure 3). PCA can be used to analyze multivariate data sets and is suitable for establishing relationships among compounds and variables. The three-dimensional plot obtained through PCA indicated that the 23 S. miltiorrhiza lines could be categorized into four distinct groups (Figure 4). This result is consistent with the clustering of chemical fingerprints obtained using the HCA. This result is attributable to the 17 common peaks all having similar area profiles within each cluster. The active compounds of all lines in the same cluster may have analogous bioactivities. This assumption is consistent with a previous study that identified three distinct clusters of S. miltiorrhiza samples based on chemical fingerprints corresponding to the grouping results of its activities against Pseudomonas aeruginosa (Kong et al., 2017).

Figures 3 and 4 indicate that the lines obtained through chromosome doubling (i.e., lines 4N2-12, 4N2-7, and 4N3-7) were categorized into the same cluster in the HCA and PCA, demonstrating that similar chemical fingerprints reflect analogous bioactivity. HCA and PCA can be used to successfully distinguish the varieties and origins of herbs with chemical fingerprints (Cheng et al., 2010; Cui et al., 2016; Ge et al., 2008). Plant phenotypes, including morphological traits, physiological responses, and metabolic compound synthesis processes, are considerably affected by genetic factors, environmental factors, and interaction between the two (Mitchell-Ol and Schmitt, 2006; O’Reilly-Wapstra et al., 2013). All S. miltiorrhiza lines used in this study were planted in the same area under the same environmental conditions, and they underwent regular cultivation management. Therefore, the environmental effects on the internal composition of the plants were minimized. The aforementioned HCA results could be obtained because the S. miltiorrhiza lines were collected from various regions throughout Taiwan, and some of the plants were obtained through chromosome doubling (Table 1). Each S. miltiorrhiza line had a distinct genetic makeup. This result was consistent with the variation in the 17 peaks common to the 23 S. miltiorrhiza lines, perhaps reflecting their diverse bioactivity.

The components with significant antioxidant and cytotoxic properties and the relationships between the fingerprints and bioactivities were investigated through MLR (Table 2). According to the first model, compounds G, B, and E were positively associated with antioxidant activity in the DPH scavenging assay, whereas compound M had a negative effect. Per the second model, compounds D (salvianolic acid B), B, G, N, and H were positively associated with antioxidant activity in the K$_3$Fe(CN)$_6$ assay, whereas compound K had a negative effect. The difference may be related to the substrate, mechanism, or experimental conditions of the antioxidant assays. However, the K$_3$Fe(CN)$_6$ reduction method and DPPH radical scavenging assay are commonly used to examine total antioxidant capacity (TAC). Csepregi et al. reported that the K$_3$Fe(CN)$_6$ reduction method and DPPH scavenging assay are structure and TAC dependent and respectively favor flavonoids and phenolic acids as substrates (Csepregi et al., 2016). The K$_3$Fe(CN)$_6$ reduction method is not suitable for thiol antioxidants, such as glutathione, and the reduction capability is measured based on only trivalent iron (Fe$^{3+}$). In addition, the reduction method is not relevant to antioxidant activity mechanistically and physiologically (Prior et al., 2005). Therefore, the two models indicated different compounds as significant contributors to the antioxidant activities. Moreover, both models identified salvianolic acid B as the active compound with significant antioxidant activity, which has been reported earlier (Zhao et al., 2008). The correlation coefficients revealed close correlations between the antioxidant activities of S. miltiorrhiza extracts and the chemical compounds. Compounds B and G were significant in both the first and second models, whereas compounds D, E, H, K, M, and N were significant in only one model. Compounds D (salvianolic acid B) has previously demonstrated antioxidant effects in vivo and in vitro (Chen et al., 2013; Wu et al., 1998). Further studies must examine the structure and function of the unidentified compound G, which was a major contributor, according to the DPPH radical scavenging assay.

Cancer types are clinically and molecularly heterogeneous (McGranahan and Swanton, 2017). In the present study, human ovarian clear cell carcinoma (TOV-21G) and alveolar lung epithelial carcinoma (A549) cells were treated with SMEs, which were evaluated for cytotoxicity. According to the third MLR model, compounds P (tanshinone IIA), B, and C were positively associated with cytotoxicity of SMEs to TOV-21G cells. Furthermore, according to the fourth model, compounds K, P (tanshinone IIA), and J (dihydrotanshinone) were positively associated with cytotoxicity to A549 cells. Our previous study revealed that tanshinone IIA and dihydrotanshinone had cytotoxic effects on A549 and TOV-21G cells (Chang et al., 2013). These tanshinone compounds are lipophilic and cytotoxic to several cancer cells in vitro (Guan et al., 2020; Zhou et al., 2020) and certain tumor xenograft models in vivo (Chang et al., 2015). Similar to the MLR models for antioxidant activity, the proposed strategy can be used to examine the cytotoxicity of constituents.

**CONCLUSION**

This work provides a general model for the exploration of the spectrum–effect relationships by assessing 171 HPLC fingerprints of S. miltiorrhiza in combination with its antioxidant and antitumor activities. Noteworthy, the use of chemometrics to rapidly and efficiently identify patterns
of effective constituents and bioactivity within the phytochemical variability of plants is feasible. The results indicated that the developed strategy can be used to identify components largely contributing to particular bioactivities and re-evaluate the efficacy of previously neglected components. The present study identified not only the primary active components of S. miltiorrhiza but also the optimal ratios of constituents, validating the method for use in the future investigation and development of herbal medicines. Collectively, this strategy can be used in future studies to identify previously neglected components that are potential pharmacological agents.

Declaration of competing Interest

The authors declared no conflict of interest in the manuscript.

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