Studies on Quality Markers of Kaihoujian Spray for Anti-Inflammation Based on Gray Correlation Analysis Strategy

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Kaihoujian spray (KHJ) was originated from the classical prescription of Miao medicine, which was commonly used for acute and chronic pharyngitis. The prescription was composed of Sophorae Tonkinensis Radix, Ardisiae Radix, Cicadae Periosis-tracum, and menthol. However, in previous literature, only clinical studies have been reported. The Quality Marker (Q-Markers) of KHJ on anti-inflammation has not been clearly elucidated. In this study, a gray correlation analysis strategy combined with network pharmacology analysis was established for the investigation of Q-Markers in KHJ. A total of 52 components were identified or tentatively characterized in KHJ, including alkaloids, saponins, bergenin, flavonoids, amino acids, and their derivatives. Furthermore, regularity of recipe composition and gray correlation analysis revealed that the correlation degree of all peaks was greater than 0.5. The ranking of correlation degree was peak 1 > 6 > 9 > 8 > 7 > 10 > 4 > 5 > 11 > 3 > 2. Among them, peaks 2, 4, 5, 6, 8, 9, and 11 were identified as anagyrine, matrine, sophocarpine, norbergenin, bengenin, 11-O-galloylbergenin, and trifolirhizin. The network pharmacology analysis revealed that EGFR, MMP9, MMP3, MMP1, and PTGS2 were the main targets of KHJ. Bergenin, matrine, sophocarpine, calycosin, and trifolirhizin were the main anti-inflammatory active ingredients in KHJ. These results proposed that bergenin, sophocarpidine, sophocarpine, and trifolirhizin could be the Q-Markers of KHJ on anti-inflammation. The process of discovering the Q-Markers would provide a promising method of quality control on KHJ.

1. Introduction

As a significant portion of traditional Chinese medicine (TCM), Miao medicine has a long history of three or four thousand years. It is generally considered to be mysterious and magical and has its own system, especially famous for its external treatment of internal diseases [1]. Miao herb formulation (MHF) is a valuable medical experience accumulated by Miao folk in their long-term production activities and the practice of fighting against diseases and injuries. They have a profound understanding of etiology, elements, disease diagnosis, treatment, and prevention and have many unique features in clinical prescription and medication [2]. Their abundant medical experience has enriched the culture and become an important part of TCM.

However, similar to TCM, MHF also has many problems, such as unclear material basis and index components. Although some MHF were included in Chinese Pharmacopoeia, the quality standards were only established on the basis of their major components. Whether the index component was related to its efficacy was still dubious.

Fortunately, the concept of Quality Marker (Q-Marker) was established by Liu et al. [3] for the development and improvement of the quality of TCM. The candidates for Q-Markers should meet these criteria [3, 4]: (1) The candidates should exist in original materials, TCM products, or formed during processing and preparation. (2) The
candidates should be unique to some herbs and not derived from other herbs. (3) The candidates should have definite chemical structures and biological activity. (4) The candidates could be qualitatively and quantitatively identified. (5) The candidates should follow the principle of TCM. For the past few years, numerous studies on Q-Markers have been published [5–14]. However, how to discover and verify the Q-Marker was still a serious challenge.

Kaihoujian spray (KHI) was originated from the classical prescription of Miao medicine, which was a commonly used Chinese patent medicine for children with acute and chronic pharyngitis, and produced by Guizhou Sanli Pharmaceutical Limited by Share Ltd. The prescription was composed of Sophorae Tonkinensis Radix, Arsidae Radix, Cicadae Periostracum, and menthol. According to the previous literature, Sophorae Tonkinensis Radix has the effects of anti-inflammation [15], antivirus [16], inhibiting bacteria [17], improving immunity [18], and so on. Arsidae Radix has the effects of bacteriostasis [19], analgesia [20], antivirus [21], and so on. Cicadae Periostracum has the effects of anti-allergic [22], antitussive and antiasthmatic [23], bacteriostatic [24], and so on. Menthol has the effects of analgesic [25], osmotic [26], and so on. Kaihoujian spray can directly act on oral mucosa and avoid first-pass effect without gastrointestinal absorption and has the clinical advantages of fast onset, high bioavailability, small side effects, short course of treatment, convenient medication, and high patient compliance [27]. At present, the Q-Markers of KHI on anti-inflammation have not been clearly elucidated. Only clinical studies have been reported in previous literature. Hence, it was necessary to develop a strategy to discover and validate the Q-Markers of KHI on anti-inflammation. In this study, a gray correlation analysis strategy combined with network pharmacology analysis was established for the investigation of Q-Markers on KHI. The results showed that bergenin, sophocarpidine, sophocarpine, and trifolirhizin should be the Q-Markers of KHI on anti-inflammation. The process of discovering the Q-Markers would provide a promising method of quality control on KHI.

2. Materials and Methods

2.1. Materials and Chemicals. The Sophorae Tonkinensis Radix (the dried root of Sophora tonkinensis Gagnep.), Arsidae Radix (the dried root of Ardisia crenata Sims.), Cicadae Periostracum (the dried shell of Cryptotympana pustulata fabricius), and menthol were provided by Guizhou Sanli Pharmaceutical Limited by Share Ltd. and identified by the Researcher Chengwang Tian. The voucher specimens (STR-2019, AR-2019, CPS-2019, and MEL-2019) were stored in herbaria at Tianjin Institute of Pharmaceutical Research, China. 

(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and lipopolysaccharides (LPSs) were purchased from Beijing Solarbio Science & Technology Co., Ltd. The Griess reagents were purchased from Tianjin Guangfu Chemical Research Institute. Dulbecco’s modified eagle’s medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Gibco Ltd. All organic solvents used in this study were of HPLC grade and purchased from Concord Technology Co., Ltd. Pure distilled water was purchased from Wahaha Group Co., Ltd. (Hangzhou, China).

2.2. Preparation of Samples. The whole prescription sample was prepared according to the method for KHI Spray Standard published by National Medical Products Administration in 2002. In a brief description, Sophorae Tonkinensis Radix (250 g), Arsidae Radix (250 g), and Cicadae Periostracum (250 g) were refluxed with pure water twice (1:10, w/v, 2 h each). After evaporation of the solvent in vacuo, ethanol was added to the residues until the ethanol content reached 80%. While kept standing for 24 hours, the solvent was filtered and evaporated in vacuo. The crude extract was mixed with menthol (1 g). Samples for regularity of recipe composition (KHI-1-14) were prepared as the above method, except for the assigned herbs. The ingredients of each sample are shown in Table 1.

2.3. HPLC/Q-ToF-MS/MS Analysis of KHI. The sample for HPLC/Q-TOF-MS/MS analysis was prepared as follows: approximately 0.1 g of the whole prescription sample (KHI-15) was dissolved and diluted to 10 mL by 40% methanol. The sample was filtered through a 0.22 μm membrane filter and then injected 10 μL into the HPLC system for analysis. Chromatographic separation was carried out on an ultimate Plus C18 column (4.6 × 250 mm, 5 μm). The mobile phase was optimized as 0.1% aqueous formic acid (A) and acetonitrile (B), and the gradient of elution was as follows: 0–15 min, 3%–8% B; 15–25 min, 8%–15% B; 25–35 min, 15%–22% B; 35–45 min, 22%–24% B; 45–75 min, 24–50% B. The flow rate was 1.0 mL/min, and the column temperature was held at 30°C. The optimum absorbed wavelength was selected as 210 nm according to the favorable resolution and multiple chromatographic peaks. The mass spectrometry analysis was obtained on a Sciex X500 R QTOF mass spectrometer equipped with an electrospray interface (ESI) source (AB Sciex, Framingham, MA, USA). Positive and negative ion modes were used for detection, the capillary voltages were 5500V and 4500V, the curtain gas was 35 PSI, and the atomizing temperature was 600°C. The mass data were achieved in the range of m/z from 50 to 1800 Da with a response value of more than 100 cps of the four highest peaks for secondary mass spectrum scaning. Data were collected and analyzed by analyst software SCIEX OS 1.4.

2.4. HPLC-DAD Analysis for Samples of Regularity of Recipe Composition. The analysis of the regularity of recipe composition samples (KHI-1–14) was performed by the Waters e2695 (United States) with a PDA detector. The gradient of elution for mobile phase, optimum absorbed wavelength, flow rate, chromatography column, and column temperature were set as HPLC/Q-TOF-MS/MS method.

2.5. Cell Culture and Cytotoxicity. RAW264.7 cells were purchased from Procell Life Science & Technology Co., Ltd.
Cells were cultured in DMEM, including 10% FBS and 2% penicillin-streptomycin solution at 37°C in humidified 5% CO2 atmosphere. Cells were made to suspension and diluted to $1 \times 10^5$ cells/mL by DMEM containing 10% FBS. Cytotoxicity of the samples was determined by MTT assay.

2.6. Inhibition of NO Production in LPS-Induced RAW264.7 Cells. RAW264.7 cells were incubated and divided into several groups. After stimulation with and without LPS (2 mg/mL) for 24 hours, the supernatant of media was collected for NO production analysis. 50 μL supernatant mixed with 50 μL Griess reagent was incubated in the dark for 10 min at 37°C. The OD value of each well was measured by a microplate reader at 540 nm. The concentration of NO was determined by the standard curve from sodium nitrite.

2.7. Statistical Analysis. The measurement data were analyzed by IBM SPSS 23.0 (USA) and expressed as means ± SD (n = 3). The data satisfying normality and homogeneity of variance were analyzed by one-way ANOVA, and the comparison between groups was performed by the LSD method. If the data do not meet the homogeneity of variance, using K independent samples nonparametric test, P < 0.05 has statistical significance.

2.8. Network Pharmacology Analysis. All the identified compounds in HPLC/Q-ToF-MS/MS analysis of KHJ were selected for the target compounds. Then these target compounds were introduced into a SwissTargetPrediction database (https://www.swisstargetprediction.ch) to predict an action target of the active compound. The TCMSP database (https://tcmspw.com/tcmsp.php) was searched for the possible targets of the active ingredients of KHJ, and then the Drugbank database (https://www.drugbank.ca/) and Uniprot database (http://www.uniprot.org) were searched for the target GeneSymbol. Taking "inflammation" as the keyword, we searched the DigSee database (http://digsee.com) to obtain the targets related to anti-inflammatory and screened the inflammatory targets with a correlation degree greater than 0.8.

The data of active compounds and inflammatory targets screened above were sorted and imported into Cytoscape 3.7.0 software to construct the active ingredient-disease target network, and the network topology was analyzed by Network Analyzer. The intersection targets of drugs and diseases were imported into the STRING database (https://www.string-db.org), the confidence value was set to 0.4, the target interaction data were exported and processed by Excel and then imported into Cytoscape 3.7.0 to realize visualization, and the topology analysis of PPI network was carried out. Through the ClueGO, gene ontology (GO) analysis was carried out on the core target of KHJ under the conditions of number of genes ≥ 3 and min percentage = 4.0. *Homo sapiens*, overlap >3, P < 0.01, and enrichment >1.5 were selected as screening conditions for GO and KEGG analysis. The pathway with lower P value and more enriched genes was screened, the GO analysis was drawn by R language ggplot2 software package, and the bubble map of KEGG analysis was drawn by Origin Pro 2021 software. Pathways with a smaller P value and more enriched genes were screened, the data of drug flavor, components, targets, and pathways were sorted out and introduced into Cytoscape 3.7.0 software to construct the network of drug flavor-component-target-pathway, and the network topology was analyzed.

### Table 1: Ingredients of each group.

| No. | Sophorae Tonkinensis Radix | Ardisiae Radix | Cicadae Periostracum | Menthol |
|-----|-----------------------------|----------------|----------------------|---------|
| KHJ1| +                           | −              | −                    | −       |
| KHJ2| −                           | +              | −                    | −       |
| KHJ3| −                           | −              | +                    | −       |
| KHJ4| −                           | −              | −                    | +       |
| KHJ5| +                           | +              | −                    | −       |
| KHJ6| +                           | −              | +                    | −       |
| KHJ7| +                           | −              | −                    | +       |
| KHJ8| −                           | +              | −                    | −       |
| KHJ9| −                           | +              | −                    | +       |
| KHJ10| −                        | −            | +                    | +       |
| KHJ11| +                          | +            | −                    | +       |
| KHJ12| +                          | +            | −                    | +       |
| KHJ13| +                          | −            | +                    | +       |
| KHJ14| −                          | +            | −                    | +       |
| KHJ15| +                          | +            | +                    | +       |

### 3. Results and Discussion

#### 3.1. Analysis of Chemical Ingredients in KHJ. The total ion chromatography (TIC) of KHJ in positive and negative modes is shown in Figure 1. A total of 52 components were identified or tentatively characterized in KHJ, including alkaloids, saponins, bergenin, flavonoids, amino acids, and their derivatives. Among them, 32 compounds (alkaloids, flavonoids, saponins, etc.) were derived from Sophorae Tonkinensis Radix, 14 compounds (coumarins, saponins, etc.) were derived from Ardisiae Radix, and 6 compounds (amino acids) were derived from Cicadae Periostracum. The identification of these compounds was mainly based on the
comparison with literature, including the retention time and fragment ion. The detailed information, including chemical formula, retention time, mass value, mass error, fragment ion, and botanical source, is shown in Table 2. The exact structures are shown in Figure 2.

3.1.1. Identification of Alkaloids in KHJ. Fifteen alkaloids from Sophorae Tonkinensis Radix were identified in KHJ. The main types of them were matrine and cytisine, and all of them could yield quasimolecular ions [M + H]⁺. Take N-methylcytisine (compound 1) as an example to illustrate the analytic process of alkaloids. The quasimolecular ion [M + H]⁺ at m/z 205.1 corresponded to the formula C₁₂H₁₆N₂O. The mass spectrum fragment ion m/z 146.0603 [M + H – C₃H₉N]⁺ was the characteristic fragment ion of the cytisine alkaloid, and m/z 108.0809 [M + H – C₆H₄NO]⁺ was generated by the cleavage and rearrangement of the parent ionic bond C₆–C₇/C₁–C₁₀. In combination with the literature [28], compound 1 was presumed to be N-methylcytisine. The mass spectrum and the cleavage rule of the N-methylcytisine are shown in Figure 3.

3.1.2. Identification of Bergenin Derivatives in KHJ. Bergenin and its derivatives are the main effective constituents of Ardisiae Radix and have the effect of relieving cough by inhibiting the cough center. In this study, seven bergenin and its derivatives from Ardisiae Radix were identified in KHJ. Take 11-O-galloylbergenin (compound 25) as an example to explain the process of identification. Compound 25 showed a favorable response in both positive ion and negative ion mode. The quasimolecular ion [M – H]⁻ at m/z 479.1 corresponded to the formula C₂₁H₂₀O₁₃. The fragment ion m/z 464.1 was formed by the loss of methyl. The ester bond of the parent ion was cleaved to form a gallic acid fragment at m/z 169.0 [M – C₆H₄O₈]⁻ and a bergenin fragment at m/z 327.1 [M – C₆H₄O₄]⁻. The fragment ion [M – H – C₁₅H₁₄O₁₀]⁻ was formed by the loss of CO₂ from the
| No. | Molecular formula | $t_R$ (min) | Calcd $(m/z)$ | Exptl $(m/z)$ | $\delta$ /ppm | Ion mode | MS/MS | Identification                      | Sources   |
|-----|-------------------|-------------|--------------|--------------|--------------|----------|-------|------------------------------------|-----------|
| 1   | C$_{12}$H$_{16}$N$_{2}$O | 4.67        | 205.1335     | 205.1328     | 3.41         | [M + H]$^+$ | 205.1314; 146.0603; 108.0809; 162.0920; 263.1753; 150.1276; 195.1491; 245.1649; 119.0492; 107.0493; 123.0442; 136.0761; 265.1914; 219.1862; 148.1123; 247.1815; 265.1999; 150.1271; 247.1820; 112.0754; 245.1649; | N-Methylcytisine | STR |
| 2   | C$_{13}$H$_{22}$N$_{2}$O$_{2}$ | 5.41        | 263.1754     | 263.1753     | 0.38         | [M + H]$^+$ | (5α/12α/12β)-Hydroxsophocarpine | STR |
| 3   | C$_{9}$H$_{11}$NO$_{3}$ | 5.49        | 182.0812     | 182.0812     | 0            | [M + H]$^+$ | Tyrosine | CPS |
| 4   | C$_{13}$H$_{24}$N$_{2}$O$_{2}$ | 5.99        | 265.1911     | 265.1914     | −1.13        | [M + H]$^+$ | (9α/5α)-Hydroxymatrine | STR |
| 5   | C$_{13}$H$_{24}$N$_{2}$O$_{2}$ | 6.61        | 265.1899     | 265.1899     | 0            | [M + H]$^+$ | 14α-Hydroxymatrine | STR |
| 6   | C$_{15}$H$_{20}$N$_{2}$O$_{2}$ | 7.03        | 245.1648     | 245.1641     | 2.86         | [M + H]$^+$ | 122.0601; 148.0760 | Anagyrine | STR |
| 7   | C$_{2}$H$_{4}$O$_{5}$ | 7.7         | 169.0143     | 169.0137     | 3.5          | [M − H]$^-$ | 125.0239; 107.0136; 249.1957; 112.0754; 245.1649 | 2,4,6-Trihydroxybenzoic | AR |
| 8   | C$_{13}$H$_{24}$N$_{2}$O$_{2}$ | 9.26        | 249.1961     | 249.1951     | 4.01         | [M + H]$^+$ | 148.1109; 176.1065; 112.0754; 103.0541; 120.0809; 247.1793; 136.1116; 179.1537; 150.1277; 247.1816; 307.2034; 148.1124; 307.2027; | Matrine | STR |
| 9   | C$_{9}$H$_{11}$NO$_{3}$ | 9.66        | 166.0863     | 166.0862     | 0.6          | [M + H]$^+$ | L-Phenylalanine | CPS |
| 10  | C$_{15}$H$_{22}$N$_{2}$O$_{2}$ | 10.56       | 247.1805     | 247.1793     | 4.85         | [M + H]$^+$ | Sophocarpine | STR |
| 11  | C$_{17}$H$_{26}$N$_{2}$O$_{3}$ | 11.12       | 307.2016     | 307.2021     | −1.62        | [M + H]$^+$ | (14β/14α)-acetyl matrine | STR |
| 12  | C$_{17}$H$_{26}$N$_{2}$O$_{3}$ | 11.8        | 307.2016     | 307.2027     | −3.58        | [M + H]$^+$ | (14β/14α)-acetyl matrine | STR |
| 13  | C$_{13}$H$_{14}$O$_{9}$ | 12.26       | 313.0565     | 313.0562     | 1            | [M − H]$^-$ | 123.046; 177.0183; 207.0293; 165.0187; 235.0246; 167.0341; 191.0346; 207.0296; 245.1655; 263.1764; 150.1278; 136.1121; 110.0602; 136.1123; 180.9101; 245.1663 | Norbergenin | AR |
| 14  | C$_{13}$H$_{22}$N$_{2}$O$_{2}$ | 12.42       | 263.1764     | 263.1764     | 0            | [M + H]$^+$ | Oxsophocarpine | STR |
| 15  | C$_{15}$H$_{20}$N$_{2}$O | 14.4        | 245.1648     | 245.1648     | 0            | [M + H]$^+$ | Sophoramine | STR |
Table 2: Continued.

| No. | Molecular formula | \( t_r \) (min) | Calcd \((m/z)\) | Exptl \((m/z)\) | \( \delta/\text{ppm} \) | Ion mode | MS/MS | Identification | Sources |
|-----|-------------------|----------------|----------------|---------------|----------------|----------|-------|---------------|---------|
| 16  | \( \text{C}_{12}\text{H}_{14}\text{N}_{2}\text{O}_{2} \) | 15.45 | 219.1128 | 219.1129 | −0.46 | \( [\text{M} + \text{H}]^+ \) | 148.0756; 160.0757; 219.1133; 261.1603; 164.0756; 149.0234; 119.0487; 142.0759; 137.0594; 109.0647; 107.0491 | N-Formylcytisine | STR |
| 17  | \( \text{C}_{13}\text{H}_{22}\text{N}_{2}\text{O}_{3} \) | 15.8 | 279.1703 | 279.1708 | −1.79 | \( [\text{M} + \text{H}]^+ \) | 149.0236; 243.1504; 261.1606; 148.0759; 191.1180; 233.1289; 160.0758; 136.0161; 137.0240; 109.0284; 192.0049; 193.0131; 234.0159; | 5α-Hydroxyosyosopercarpine | STR |
| 18  | \( \text{C}_{10}\text{H}_{13}\text{NO}_{3} \) | 17.58 | 196.0968 | 196.0969 | −0.51 | \( [\text{M} + \text{H}]^+ \) | 142.0759; 191.1180; 261.1606; 148.0759; 137.0594; 109.0647; 107.0491 | N-Acetyldopamine | CPS |
| 19  | \( \text{C}_{15}\text{H}_{22}\text{N}_{2}\text{O}_{3} \) | 18.72 | 279.1703 | 279.1714 | −3.94 | \( [\text{M} + \text{H}]^+ \) | 148.0756; 243.1504; 261.1606; 148.0759; 191.1180; 233.1289; 160.0758; 108.0211; 136.0161; 137.0240; 109.0284; 192.0049; 193.0131; 234.0159; | 12β-Hydroxyosyosopercarpine | STR |
| 20  | \( \text{C}_{13}\text{H}_{16}\text{N}_{2}\text{O}_{2} \) | 18.88 | 233.1285 | 233.1289 | −1.72 | \( [\text{M} + \text{H}]^+ \) | 142.0759; 191.1180; 261.1606; 148.0759; 137.0594; 109.0647; 107.0491 | N-Acetylcytisine | STR |
| 21  | \( \text{C}_{7}\text{H}_{6}\text{O}_{3} \) | 19.21 | 137.0244 | 137.0238 | 4.38 | [M \( - \)H] \( ^− \) | 142.0759; 191.1180; 261.1606; 148.0759; 137.0594; 109.0647; 107.0491 | 3,4-Dihydroxybenzaldehyde | CPS |
|     |                   | 21.31 | 327.0722 | 327.0714 | 2.44 | [M \( - \)H] \( ^− \) | 142.0759; 191.1180; 261.1606; 148.0759; 137.0594; 109.0647; 107.0491 | 3,4-Dihydroxybenzaldehyde | CPS |
| 22  | \( \text{C}_{14}\text{H}_{16}\text{O}_{9} \) | | | | | [M \( + \)H] \( ^+ \) | | Bergenin | AR |
|     |                   | 21.37 | 329.0867 | 329.0864 | 0.91 | [M \( + \)H] \( ^+ \) | 142.0759; 191.1180; 261.1606; 148.0759; 137.0594; 109.0647; 107.0491 | Bergenin | AR |
| 23  | \( \text{C}_{21}\text{H}_{20}\text{O}_{9} \) | 28.5 | 415.1035 | 415.1046 | −2.65 | [M \( - \)H] \( ^− \) | 267.0655; 295.0607; 417.1185; 399.1076; 297.0761; 381.0976; 153.0178; | Bayin | STR |
| 24  | \( \text{C}_{27}\text{H}_{30}\text{O}_{13} \) | 28.74 | 563.1759 | 563.1761 | −0.36 | [M \( + \)H] \( ^+ \) | 267.0655; 295.0607; 417.1185; 399.1076; 297.0761; 381.0976; 153.0178; | Sophoraflavone A | STR |
|     |                   | 33.58 | 481.0977 | 481.0969 | 1.66 | [M \( + \)H] \( ^+ \) | 267.0655; 295.0607; 417.1185; 399.1076; 297.0761; 381.0976; 153.0178; | Sophoraflavone A | STR |
| 25  | \( \text{C}_{21}\text{H}_{20}\text{O}_{13} \) | | | | | [M \( - \)H] \( ^− \) | | 11-O-Galloylbergenin | AR |
|     |                   | 33.6  | 479.0831 | 479.0805 | 0.83 | [M \( - \)H] \( ^− \) | 267.0655; 295.0607; 417.1185; 399.1076; 297.0761; 381.0976; 153.0178; | 11-O-Galloylbergenin | AR |
| 26  | \( \text{C}_{20}\text{H}_{22}\text{N}_{2}\text{O}_{6} \) | 35.04 | 387.1551 | 387.1545 | 1.55 | [M \( + \)H] \( ^+ \) | 267.0655; 295.0607; 417.1185; 399.1076; 297.0761; 381.0976; 153.0178; | \((2R,3S)-2-(3′,4′-Dihydroxyphenyl)-3-acetylamino-7-(N-acetyl-2″-aminoethyl)-1,4-benzodioxane\) | CPS |
| 27  | \( \text{C}_{20}\text{H}_{22}\text{N}_{2}\text{O}_{6} \) | 36.77 | 387.1551 | 387.1544 | 1.81 | [M \( + \)H] \( ^+ \) | 267.0655; 295.0607; 417.1185; 399.1076; 297.0761; 381.0976; 153.0178; | \((2R,3S)-2-(3′,4′-Dihydroxyphenyl)-3-acetylamino-6-(N-acetyl-2″-amino-1″-hydroxyethyl)-1,4-benzodioxane\) | CPS |
| No. | Molecular formula | $t_R$ (min) | Calcd (m/z) | Exptl (m/z) | $\delta$ /ppm | Ion mode | MS/MS Identification | Sources |
|-----|-------------------|-------------|-------------|-------------|---------------|----------|----------------------|---------|
| 28  | C$_{21}$H$_{20}$O$_{10}$ | 37.21 | 433.1129 | 433.1136 | −1.62 | [M + H]$^+$ | 271.0604; 164.9305; 243.0655 | Vitexin STR |
|     |                   | 37.27 | 431.0984 | 431.0996 | −2.78 | [M − H]$^-$ | 269.0460; 186.9376; 119.9454 | |
| 29  | C$_{28}$H$_{34}$O$_{13}$ | 39.35 | 579.2083 | 579.2099 | −2.76 | [M − H]$^-$ | 417.1562; 181.0501 | Syringaresinol-4-O-β-D-glucopyranoside (2R,3S)-2-(3′,4′-Dihydroxyphenyl)-3-acetylamino-7-(N-acetyl-2″-aminoethyl)-1,4-benzodioxane (2R,3S)-2-(3′,4′-Dihydroxyphenyl)-3-acetylamino-7-(N-acetyl-2″-aminoethyl)-1,4-benzodioxane |
|     |                   | 40.35 | 407.1219 | 407.1218 | 0.25 | [M + Na]$^+$ | 150.0549; 249.0551; 284.0926; 151.0391; 209.0452; 181.0500; 247.0604 | CPS |
| 30  | C$_{20}$H$_{20}$N$_{2}$O$_{6}$ | 40.35 | 407.1219 | 407.1218 | 0.25 | [M + Na]$^+$ | 150.0549; 249.0551; 284.0926; 151.0391; 209.0452; 181.0500; 247.0604 | |
|     |                   | 41.64 | 385.1394 | 385.14 | −1.56 | [M + H]$^+$ | 150.0549; 249.0551; 284.0926; 151.0391; 209.0452; 181.0500; 247.0604 | 11-O-Vanilloyl-bergenin AR |
| 31  | C$_{20}$H$_{20}$N$_{2}$O$_{6}$ | 41.64 | 385.1394 | 385.14 | −1.56 | [M + H]$^+$ | 150.0549; 249.0551; 284.0926; 151.0391; 209.0452; 181.0500; 247.0604 | 11-O-Vanilloyl-bergenin AR |
|     |                   | 44.42 | 479.1184 | 479.1187 | −0.63 | [M + H]$^+$ | 150.0549; 249.0551; 284.0926; 151.0391; 209.0452; 181.0500; 247.0604 | 11-O-Vanilloyl-bergenin AR |
| 32  | C$_{22}$H$_{22}$O$_{12}$ | 44.54 | 477.1039 | 477.1018 | 4.4 | [M − H]$^-$ | 269.0813; 254.0578 | |
|     |                   | 44.54 | 477.1039 | 477.1018 | 4.4 | [M − H]$^-$ | 269.0813; 254.0578 | 11-O-Syringyl-bergenin AR |
| 33  | C$_{23}$H$_{24}$O$_{13}$ | 44.81 | 509.129 | 509.1303 | −2.55 | [M + H]$^+$ | 209.0454; 275.0547; 153.0444 | 11-O-Syringyl-bergenin AR |
|     |                   | 47.5 | 431.1337 | 431.1340 | −0.7 | [M + H]$^+$ | 269.0813; 254.0578 | Ononin STR |
| 34  | C$_{23}$H$_{24}$O$_{13}$ | 47.5 | 431.1337 | 431.1340 | −0.7 | [M + H]$^+$ | 269.0813; 254.0578 | Ononin STR |
|     |                   | 49.03 | 509.129 | 509.129 | 0 | [M + H]$^+$ | 275.0561; 192.0059; 207.0297; 234.0166 | 11-O-(3′,4′-Dimethyl-galloyl) bergenin AR |
| 35  | C$_{23}$H$_{24}$O$_{13}$ | 49.18 | 507.1144 | 507.112 | 4.73 | [M − H]$^-$ | 275.0561; 192.0059; 207.0297; 234.0166 | 11-O-(3′,4′-Dimethyl-galloyl) bergenin AR |
|     |                   | 49.18 | 507.1144 | 507.112 | 4.73 | [M − H]$^-$ | 275.0561; 192.0059; 207.0297; 234.0166 | 11-O-(3′,4′-Dimethyl-galloyl) bergenin AR |
| 36  | C$_{54}$H$_{80}$O$_{24}$ | 49.91 | 1143.5563 | 1143.5576 | −1.17 | [M + Na]$^+$ | 421.3476; 615.3938 | Subprosides V STR |
|     |                   | 50.58 | 1099.5294 | 1099.5283 | 1 | [M + Na]$^+$ | 421.3476; 615.3938 | — |
| 37  | C$_{52}$H$_{82}$O$_{23}$ | 50.65 | 1075.5331 | 1075.5309 | 2.05 | [M − H]$^-$ | 1076.5404; 1075.5372; 943.4975 | Ardisicrenoside H AR |
|     |                   | 51.21 | 285.0758 | 285.0762 | −1.34 | [M + H]$^+$ | 1076.5404; 1075.5372; 943.4975 | — |
| 38  | C$_{16}$H$_{13}$O$_{5}$ | 51.32 | 283.0612 | 283.0615 | −1.06 | [M − H]$^-$ | 225.0547 | Calycosin STR |
|     |                   | 51.32 | 283.0612 | 283.0615 | −1.06 | [M − H]$^-$ | 225.0547 | Calycosin STR |
Table 2: Continued.

| No. | Molecular formula | tR (min) | Calculd (m/z) | Exptl (m/z) | δ /ppm | Ion mode | MS/MS | Identification | Sources |
|-----|-------------------|----------|---------------|-------------|--------|----------|-------|---------------|---------|
| 39  | C_{22}H_{22}O_{10} | 53.82    | 469.1107      | 469.11      | 2.28   | [M + Na]^+ |       | Trifolirhizin  | STR     |
|     |                   | 53.92    | 491.1195      | 491.1198    | −0.61  | [M + HCOOH−H]^− |     |               |         |
| 40  | C_{23}H_{24}O_{12} | 53.89    | 491.1195      | 491.1182    | 2.65   | [M − H]^−   |       | 11-O-3,5-Dimethoxybenzoyl bergenin | AR |
|     |                   | 56.77    | 975.5159      | 975.5179    | −2.05  | [M + H]^+   |       | Kudzusaponin A3 | STR |
| 41  | C_{48}H_{78}O_{20} | 56.82    | 973.5014      | 973.5052    | −3.9   | [M − H]^−   |       | Ardisicrenoside B | AR |
|     |                   | 57.69    | 1085.5494     | 1085.5489   | 0.46   | [M + Na]^+ |       |               |         |
| 42  | C_{32}H_{46}O_{22} | 57.74    | 1107.5593     | 1107.5641   | −4.33  | [M + HCOOH−H]^− |     |               |         |
|     |                   | 59.02    | 959.521       | 959.5229    | −1.98  | [M + H]^+   |       | Subproside II methyl ester | STR |
| 43  | C_{48}H_{78}O_{19} | 63.21    | 1113.5464     | 1113.5452   | 1.08   | [M + Na]^+ |       | Trifolirhizin 6′-monoaacetate | STR |
|     |                   | 64.75    | 1089.5487     | 1089.5336   | −4.50  | [M − H]^−   |       | Ardisicrenoside G | AR |
| 44  | C_{53}H_{86}O_{22} | 64.74    | 1105.5436     | 1105.5469   | −2.98  | [M + HCOOH−H]^− |     |               |         |
|     |                   | 65.18    | 1097.5487     | 1097.5481   | 0.55   | [M + Na]^+ |       | Ardisiacrispin A | AR |
| 45  | C_{53}H_{86}O_{23} | 65.26    | 1119.5593     | 1119.5625   | −2.86  | [M + HCOOH−H]^− |     |               |         |
|     |                   | 65.8     | 269.0808      | 269.0812    | −1.49  | [M + H]^+   |       | Ardisicrenoside N | AR |
| 46  | C_{16}H_{12}O_{4}  | 64.74    | 1083.5312     | 1083.531    | 0.18   | [M + Na]^+ |       | Formononetin | STR |
|     |                   | 65.18    | 1097.5487     | 1097.5481   | 0.55   | [M + Na]^+ |       |               |         |
| 47  | C_{53}H_{86}O_{22} | 65.26    | 267.0663      | 267.0663    | 0      | [M − H]^− |       |               |         |
|     |                   | 65.8     | 269.0808      | 269.0812    | −1.49  | [M + H]^+   |       | 8-O-Methylretusin | STR |
| 48  | C_{17}H_{14}O_{5}  | 65.94    | 299.0914      | 299.0928    | −4.68  | [M + H]^+   |       |               |         |
| 49  | C_{17}H_{14}O_{5}  | 56.83    | 299.0914      | 299.0923    | −3.01  | [M + H]^+   |       | Pterocarpin | STR |
|     |                   | 66.83    | 299.0914      | 299.0923    | −3.01  | [M + H]^+   |       |               |         |
3.1.3. Identification of Amino and Its Derivatives in KHJ. Seven nitrogenous compounds from Cicadae Periostracum were identified from KHJ and speculated to be amino acids and acetyldopamine dimers. Amino acid was the main inflammatory and antioxidant component of Cicadae Periostracum [24]. Take (2R,3S)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(N-acetyl-2′-aminoethyl)-1,4-benzodioxane (compound 26) and (2R,3S)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-6-(N-acetyl-2′-amino-1′-hydroxethyl)-1,4-benzodioxane (compound 27) as examples for the interpretation of structure analysis. Compounds 26 and 27 were isomers with the molecular formula of C26H26O11 based on the quasimolecular ion [M + H]⁺ at m/z 313.1. Gallic acid fragment. Demethylbergenin fragment m/z 313.1 [C13H13O9]⁺ was produced due to the loss of methyl by the bergenin fragment. The bergenin fragment was cleaved to produce fragments m/z 235.0, m/z 211.0, and 193.0. Combined with literature [29], compound 25 was speculated to be 11-O-galloylbergenin. The mass spectrum cracking rule of compound 25 is shown in Figure 4.

3.1.4. Identification of Flavonoids in KHJ. Eight flavonoids from Sophorae Tonkinensis Radix were identified in KHJ, including dihydroisoflavones and pterocarpin. Compared with dihydroisoflavones, pterocarpin compounds were more compact in structure and less prone to RDA rearrangement. It is reported that flavonoids from Sophorae Tonkinensis Radix have an effect on anti-inflammatory, antibacterial, and other biological activities. Trifolirhizin (compound 39) was used as a sample to expound the process of analysis. The formula C48H78O18 was confirmed due to the quasimolecular ion [M + Na]⁺ at m/z 514.3. The MS² fragmental ions at m/z 328.1, m/z 269.1, and m/z 206.1 were obtained due to the loss of C₃H₆NO, C₇H₁₀NO₂, and C₈H₉NO₃. According to the retention time in literature [20], compounds 26 and 27 were identified. The mass spectrum and possible cleavage pathways of compound 26 are shown in Figure 5.

3.1.5. Identification of Saponin in KHJ. Nine saponins were identified from KHJ. Among them, subprosides V, subproside II methyl ester, soyasaponin I, and kudzusaponin A3 were isolated from Sophorae Tonkinensis Radix, and ardisicrenoside B, ardisicrenoside H, ardisicrenoside G, ardisicrenoside N, and ardisiacrispin A were isolated from Ardisia Radix. Take soyasaponin I (compound 52) as an example to explain the process of compound analysis. The quasimolecular ion [M + H]⁺ at m/z 943.5 corresponded to the formula C₄₈H₇₈O₁₈. The fragment at m/z 797.5, 635.4, and 599.4 was produced by the successive loss of a rhamnose residue, galactose residue, and two H₂O from the parent ion. The fragment at m/z 441.3 and 423.4 was formed by the successive loss of H₂O from aglycone. Thus, compound 52 was speculated as soyasaponin I. The mass spectrum cracking rule of compound 52 is shown in Figure 7.

3.2. Cytotoxicity of KHJ against RAW264.7 Cells. The cytotoxicity of the regularity of recipe composition samples and the whole prescription sample was determined by MTT assay [30]. The results showed that KHJ had no cytotoxicity on RAW 264.7 cells at the doses of 1–100 μg/mL. The cell survival rate of KHJ-2, KHJ-4, and KHJ-9 was significantly lower than that of the blank group (P < 0.05) at the dose of 200 μg/mL, indicating that KHJ-2, KHJ-4, and KHJ-9 at the concentration of 200 μg/mL could inhibit the proliferation of RAW264.7 cells.

3.3. Inhibition Effect of KHJ on NO Production in LPS-Induced RAW 264.7 Cells. As shown in Figure 8, LPS-induced RAW264.7 cells can significantly promote the production of NO, and the content of NO in KHJ-4 had no significant difference compared with that in the model group (P > 0.05). Other groups could inhibit the release of NO in RAW264.7 cells to different degrees (P > 0.05).
(a)

Figure 2: Continued.
Figure 2: Continued.
Figure 2: Continued.
The IC value was calculated by IBM SPSS 23.0 and converted into the concentration of the samples. As shown in Table 3, KHJ-2, KHJ-9, KHJ-5, KHJ-11, and KHJ-1 groups had a stronger anti-inflammatory effect in vitro. These results indicated that the anti-inflammatory active ingredients of KHJ mainly came from Sophorae Tonkinensis Radix and Ardisiae Radix.

3.4. Gray Correlation Analysis. Gray relational analysis (GRA) was a quantitative description and comparison method for the development and change of a system. Its basic idea was to judge whether the relationship was close by determining the geometric shape similarity between the reference data column and several comparative data columns, which reflects the correlation degree between

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**Figure 2:** Chemical structures identified in KHJ.
Figure 3: The mass spectrum and the cleavage rule of the N-methylcytisine.

Figure 4: Continued.
Figure 4: The mass spectrum and the cleavage rule of 11-O-galloylbergenin.

Figure 5: Continued.
Figure 5: The mass spectrum and the cleavage rule of \((2R,3S)\)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(N-acetyl-2″-aminoethyl)-1,4-benzodioxane.

Figure 6: Continued.
Figure 6: The mass spectrum and the cleavage rule of trifolirhizin.

Figure 7: Continued.
Figure 7: The mass spectrum and the cleavage rule of soyasaponin I.

Figure 8: Inhibition effect of KHJ on NO production in LPS-induced RAW 264.7 cells.
the curves. In this study, the spectrum-effect relationship of KHJ was studied through the GRA method. A total of 11 main peaks were selected according to the HPLC-DAD analysis for the regularity of recipe composition samples (Figure 9). The anti-inflammatory activity score of each sample was taken as a reference column, and the

| No. | IC50 value (crude drug μg/mL) | Score of anti-inflammatory effect |
|-----|-------------------------------|----------------------------------|
| KHJ1 | 742.02                        | 11                               |
| KHJ2 | 306.67                        | 15                               |
| KHJ3 | 1674.16                       | 3                                |
| KHJ4 | —                             | —                                |
| KHJ5 | 683.86                        | 13                               |
| KHJ6 | 875.4                         | 8                                |
| KHJ7 | 990.67                        | 6                                |
| KHJ8 | 821.13                        | 9                                |
| KHJ9 | 450.51                        | 14                               |
| KHJ10 | 2180.11                      | 2                                |
| KHJ11 | 719.08                       | 12                               |
| KHJ12 | 941.07                       | 7                                |
| KHJ13 | 1303.93                      | 5                                |
| KHJ14 | 1456.57                      | 4                                |
| KHJ15 | 788.03                       | 10                               |

Table 3: Anti-inflammatory effect of KHJ.

Table 4: Peak area and correlation analysis results of each chromatographic peak.

| No. | Peak area                                      | Correlation degree |
|-----|-----------------------------------------------|--------------------|
| KHJ1  | 337675 3250741 7883555 1361583 6562137        | 0.721              |
| KHJ2  | 131096 3549827 11948092 655154 672761 4204828 | 0.55               |
| KHJ3  | 269484 338361 2194995 3945104 655154 672761 4204828 | 0.577            |
| KHJ5  | 367008 131227 3761086 666981 3197515 2293113 1772313 | 0.648            |
| KHJ7  | 287472 2647413 6463130 1140807 5399129 2891330 | 0.645            |
| KHJ8  | 225653 15812017 425171 493058 24402042 2135026 3281601 | 0.713            |
| KHJ9  | 124643 3316416 1118286 2705511 2905159 7515400 2453783 | 0.698            |
| KHJ10 | 1591425 521181 5757911 2261501 409749 424601 3120952 20053990 3172558 2355283 1200859 | 0.657            |
| KHJ11 | 254038 104866 1791561 3318848 586303 448102 3736736 17817825 3334396 1317748 1176199 | 0.638            |
| KHJ12 | 449347 121253 4832987 952655 4008906 2999136 2504868 | 0.633            |
| KHJ13 | 175460 13509058 402603 565591 22001498 2226591 3614359 | 0.638            |
| KHJ14 | 244968 566730 5256412 2078015 406265 369635 2921097 19315474 2644608 2116705 1088261 | 0.638            |
| KHJ15 | 788.03 3250741 7883555 1361583 6562137        | 0.638            |

Figure 9: Eleven main peaks in KHJ.
correlation degree was calculated after the original data was treated with the dimensionless standard. The results of peak area and spectrum-effect correlation analysis are shown in Table 4.

As shown in Table 4, the correlation degree of all peaks was greater than 0.5. This indicated that ingredients in KHJ were acting in synergy. The ranking of correlation degree was peak 1 > 6 > 9 > 8 > 7 > 10 > 4 > 5 > 11 > 3 > 2. Among them, peaks 2, 4, 5, 6, 8, 9, and 11 were identified as anagyrine, matrine, sophocarpine, norbergenin, ben- genin, 11-O-galloylbergenin, and trifolirhizin through HPLC/Q-Tof-MS/MS analysis. These ingredients could be candidates for the Q-Markers of KHJ on anti-inflammation.

3.5. Network Pharmacological Analysis. As shown in Figure 10, the active ingredient of the drug-potential target was visualized by Cytoscape 3.7.0. The key active ingredients, which are higher than the average value and the core target of KHJ, were imported into the STRING database and processed by Cytoscape 3.7.0 to obtain the PPI network (Figure 11). The network consists of 65 nodes and 293 edges. The targets whose values were greater than the average degree value were as follows: GAPDH, EGFR, TNF, PTGS2, MMP 9, CCND1, ESR1, AR, PLAU, MMP3, AGTR1, MMP1, ADAM17, IL2, CTSB, MMP7, NOS2, PARP1, MME, and TLR9. Metascape database (https://metascape.org) was used for GO enrichment analysis and KEGG analysis of 20 key targets. The 20 key targets

![Figure 10: Ingredient-target network.](image1)

![Figure 11: PPI network.](image2)
The Most Enriched GO Terms

- response to UV-A
- regulation of inflammatory response
- response to UV
- cellular response to UV-A
- collagen catabolic process
- positive regulation of inflammatory response
- neuroinflammatory response
- response to light stimulus
- positive regulation of defense response
- positive regulation of response to light
- cellular response to abiotic stress
- cellular response to environmental stimulus
- cellular response to UV
- collagen metabolic process
- epithelial cell proliferation
- nuclear envelope
- nuclear membrane
- extracellular matrix
- membrane raft
- membrane microdomain
- metalloendopeptidase activity
- endopeptidase activity
- metallopeptidase activity
- peptidase activity, acting on...
- peptidase activity

GeneNumber

BP
CC
MF

(a)

Figure 12: Continued.
Figure 12: GO enrichment analysis.

Figure 13: KEGG analysis.
were mainly involved in biological functions such as ultraviolet response, inflammation regulation, collagen catabolism process, light stimulation response, nerve inflammation response, and external stimulation response by influencing the activities of metalloendopeptidase, serine proteolytic enzyme, and serine-type endopeptidase. The results are shown in Figure 12(a). The 20 key targets were analyzed for GO enrichment by ClueGO. As shown in Figure 12(b), 20 key targets of KHJ were mainly involved in the nitric oxide synthase activity signal transduction pathway, ultraviolet radiation response signal pathway, nerve inflammation response, and collagen catabolism process.

The Metascape database was used for KEGG analysis, involving 61 entries, in which the cancer pathway, prostate cancer pathway, and interleukin-17 signal pathway were enriched with more genes and smaller $P$ value. These results are shown in Figure 13.

The data of compounds, targets, and pathways were imported into Cytoscape 3.7.0 to obtain a network, which contains 58 nodes and 175 edges, and the nodes increased with the degree value. The results suggested that KHJ may exert an anti-inflammatory effect through multicomponent and multitarget. Bergenin, matrine, sophocarpine, calycosin, and trifolirhizin were the main anti-inflammatory active ingredient in KHJ. The main targets of KHJ were EGFR, MMP9, MMP3, MMP1, and PTGS2. The results are shown in Figure 14.

4. Conclusion

Miao medicine was an important part of TCM. However, similar to TCM, the lack of quality standards also seriously restricted the standardization and modernization of Miao medicine. The proposal of Q-Marker pointed out the direction for the quality research of TCM. However, how to discover and identify the Q-Marker was still a great challenge for TCM and Miao medicine.

In this paper, a gray correlation analysis strategy combined with network pharmacology analysis was proposed to investigate the Q-Markers of KHJ. The results show that bergenin, sophorarpidine, sophocarpine, and trifolirhizin could be regarded as the Q-Markers of KHJ on anti-inflammatory. The process of discovering the Q-Markers would provide a promising method of quality control on KHJ. Nevertheless, the specific contribution of each Q-Marker in the formulation had not been clarified, which needs to be further investigated.

Data Availability

All data used to support the findings of this study are included within this paper.

Conflicts of Interest

All the authors declare that there are no conflicts of interest.

Authors’ Contributions

Jinpeng Chen, Chengwang Tian, and Tiejun Zhang proposed the concept and design of the study. Jinpeng Chen, Yi Liu, and Xiaohong Gai executed the collecting and analysis of data. Qing Ye and Siyu Zhou accomplished the literature search. Jinpeng Chen drafted the main manuscript, while Chengwang Tian and Tiejun Zhang were responsible for
editing and providing critical revision. All authors have read and agreed to the published version of the paper.

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