Acta Pharmaceutica Sinica B 2016;6(2):148–157

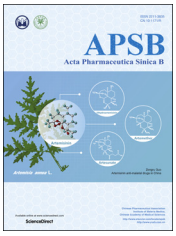
HOSTED BY

ELSEVIER

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

# Flavonoid profiling of a traditional Chinese medicine formula of Huangqin Tang using high performance liquid chromatography

**Tao Li[a,b,†], Shuaixing Zhuang[a,†], Yiwei Wang[c], Yanli Wang[a], Weihao Wang[a], Huihui Zhang[a], Li Chen[a], Dunfang Wang[a], Zhongming Zhou[a], Weipeng Yang[a,*]**

[a]*Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China*
[b]*Experimental Research Center, China Academy of Chinese Medical Sciences, Beijing 100700, China*
[c]*China Food and Drug Administration, Beijing 100053, China*

Received 4 November 2015; received in revised form 23 December 2015; accepted 24 December 2016

**KEY WORDS**

Radix Scutellariae;
Flavonoids;
HPLC fingerprinting analysis;
Multiple-component quantitative analysis;
Paw edema;
Carrageenin;
Antipyretic;
Anti-inflammatory

**Abstract** The quality control processes for herbal medicines have been problematic. Flavonoids are the major active components of Huangqin Tang (HQT, a traditional Chinese medicine formula). In this study, we used a combinative method approach consisting of chromatographic fingerprinting (high performance liquid chromatography; HPLC), quantitative methods and a pharmacodynamic evaluation model to analyze the flavonoids of HQT obtained from different sources. Ten batches of HQT were analyzed by the HPLC fingerprinting method and 26 common peaks were detected, of which 23 peaks corresponded with the chemical profile of HQT. In addition, 11 major compounds were identified by LC–MS analysis (liquid chromatography–tandem mass spectrometer; LC–$MS^n$) and quantified by the HPLC quantitative method approach. The studied 10 batches of HQT were found to be homogeneous in their composition with a similarity between 0.990 and 1.000. The distribution of the 11 identified compounds was found to be very similar among the batches. Only slight pharmacodynamic differences were detected between the different batches, confirming the homogeneity of HQT. The results of this study prove that the combination of chromatographic fingerprinting and quantitative analysis can be readily used for comprehensive quality control of herbal medicines.



*Abbreviations:* CFDA, China Food and Drug Administration; HPLC, high performance liquid chromatography; HQT, Huangqin Tang; ICH, International Conference on Harmonization; LC–$MS^n$, liquid chromatography–tandem mass spectrometer; LLOD, linearity, lower limit of detection; LLOQ, lower limit of quantification; PCA, principal component analysis; RSD, relative standard deviation; *S/N*, signal-to-noise ratio; TCM, traditional Chinese medicine.

*Corresponding author. Tel.: +86 10 64093014; fax: +86 10 64013996.
E-mail address: hrbywp@sina.com (Weipeng Yang).
†These authors made equal contributions to this work.

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

http://dx.doi.org/10.1016/j.apsb.2016.01.001

## 1. Introduction

Traditional Chinese medicine (TCM), especially herbal medicine, is gaining increasing attention worldwide for its millennia-old practices and its potential all-natural therapeutic applications. Huangqin Tang (HQT), a well-known Chinese medicine formula, is a combination of four herbal medicines 3:2:2:2 by weight, namely Radix Scutellariae (*Scutellaria baicalensis* Georgi), Radix Paeoniae Alba (*Paeonia lactiflora* Pall.), Radix et Rhizoma Glycyrrhizae (*Glycyrrhiza uralensis* Fisch., *Glycyrrhiza inflata* Bat. or *Glycyrrhiza glabra* L.) and Fructus Jujubae (*Ziziphus jujuba* Mill). HQT has traditionally been used for the treatment of colds and gastrointestinal diseases with symptoms of fever, abdominalgia and diarrhea[1,2]. Recently, some studies have reported that PHY906, an extract derived from HQT, reduces gastrointestinal toxicity and enhances the anti-tumor effect of some anti-cancer drugs[3,4]. This extract has been studied in five clinical trials with patients suffering from different types of cancer in both the US and Taiwan with very encouraging results. Radix Scutellariae, the chief ingredient of HQT, has been widely used in TCM prescriptions[5]. It is known to be effective in the treatment of fever, inflammation, cancers and other gastrointestinal diseases[6–9]. The major functional constituents of Radix Scutellariae are flavonoid glycosides (*e.g.*, baicalin, wogonoside and oroxylin-A-glucoside) and flavonoids (*e.g.*, baicalein, wogonin and oroxylin-A)[10–12]. Phytochemical studies have shown that the main constituents of the other three herbs (Radix Paeoniae Alba, Radix et Rhizoma Glycyrrhizae and Fructus Jujubae) are flavones, isoflavones, terpenoids, volatile oils and polysaccharides with a wide range of pharmacological properties[13–15], such as anti-inflammatory, analgesic, antitussive, tumor suppressor and immunomodulatory[16–19]. Flavonoids are the important effective components from HQT.

Generally, the chemical composition of herbal formulations may vary greatly due to differences in plant origins, cultivation areas and practices, climate conditions and processing protocols among others[20–22]. This leads to wide disparities in quality among different samples. An effective and feasible method should take the complexity and variability of the chemical constituents of herbal medicines into consideration simultaneously. However, previous studies addressing HQT using fingerprinting analysis failed to include enough quality control markers or had a limited study sample population[23,24]. Here we use a combinatorial approach with chromatographic fingerprinting analysis, a multiple-component quantitative analysis and animal pharmacology to comprehensively assess the quality of different HQT samples.

## 2. Materials and methods

### 2.1. Chemicals and herbal materials

The reference standards for baicalin, wogonoside, oroxylin-A-glucoside, baicalein, wogonin, oroxylin-A, liquiritin, liquiritigenin, isoliquiritoside, isoliquiritigenin, liquiritin apioside and isoliquiritin apioside were purchased from Zelang Pharmaceuticals (Nanjing, China). The purity of these compounds was higher than 98% by HPLC analysis based on a peak area normalization method. Aspirin (Scientific Research Special) and carrageenin (Sigma Life Science), both in pure powdered form, were also used. Analytical-grade ethanol and acetic acid were purchased from Beijing Chemical Factory (Beijing, China). HPLC-grade methanol and acetonitrile were obtained from Fisher (Fisher Scientific, USA). Purified water was purchased from Wahaha Group (Hangzhou, China).

Crude Radix Scutellariae from three origins that met the requirements of Beijing Food and Drug Administration was collected from different pharmacies in Beijing. Crude Radix Rhizoma Glycyrrhizae, Radix Paeoniae and Fructus Jujubae (Herbal Pieces Co., Ltd.; originally from Anhui, Neimeng and Hebei, respectively) were collected from Beijing Wukesong Clinic. The source and labeling of the medicines is summarized in Table 1. All herbal medicines were identified by Professor Xianduan Li of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences and voucher specimens were deposited in the institute. HPLC analysis was used to confirm that the content of baicalin in crude Radix Scutellariaeis is $14.9 \pm 2.7\%$ in accordance with the quality level request of 4.0% established in the *Chinese Pharmacopoeia* 2010[5].

### 2.2. Preparation of HQT granule, HQT sample solutions and standard solutions

Ten batches of crude Radix Scutellariae (9 g) and one batch of Radix et Rhizoma Glycyrrhizae (6 g), Radix Paeoniae (6 g) and Fructus Jujubae (6 g) were weighed and thoroughly soaked in water for 30 min. For the first decoction, 10-fold water (1:10 *w/v*) was added to the crude drugs and the mixture was boiled for 1.5 h, after which the decoction was filtered. At this point, 8-fold water (1:8 *w/v*) was added to the crude drug residue. Then the mixture was boiled for additional 1 h. Following filtration, the decoction was combined with previous one and the sample was dried in a vacuum oven at 60 °C into a granulated powder which was

**Table 1** Huangqin Tang samples used in the study and their calculated similarity values.

| Sample No. | Batch No. | Collection site of Radix Scutellariae | Origin of Radix Scutellariae | Ratio extraction (%) | Similarity |
|---|---|---|---|---|---|
| S1 | 20120427 | Beijing Tianheng Pharmacy | Neimeng | 39.39 | 1.000 |
| S2 | 20120519 | Beijing Tongzhitang Pharmacy | Neimeng | 33.22 | 0.998 |
| S3 | 20120512 | Beijing Tongrentang Pharmacy | Neimeng | 32.56 | 0.990 |
| S4 | 20120522 | Beijing Hedantang Pharmacy | Neimeng | 32.67 | 0.997 |
| S5 | 20120523 | Beijing Jinxiang Pharmacy | Hebei | 32.11 | 0.996 |
| S6 | 20120519 | Beijing Yongantang Pharmacy | Hebei | 33.78 | 0.999 |
| S7 | 20120427 | Beijing Xingainian Pharmacy | Hebei | 38.89 | 0.999 |
| S8 | 20120512 | Beijing Wukesong Clinic | Hebei | 36.00 | 0.998 |
| S9 | 20120523 | Beijing Jingzhitang Pharmacy | Shanxi | 33.67 | 0.999 |
| S10 | 20120426 | Beijing Jinglongtang Pharmacy | Shanxi | 33.56 | 0.999 |

packaged and stored at 4 °C for future use. Ratio extraction was calculated using the following formula:

$$\text{Ratio extraction}\ (\%) = W_1/W_2 \times 100 \quad (1)$$

where $W_1$ is the weight of the dried HQT granules and $W_2$ is the weight of the crude HQT mixture.

Dried HQT granules (approximately 0.50 g) were ground into a powder (approximately 40 meshes) and weighed. For phytochemical analysis, the HQT powder was extracted with 50 mL of 75% (*w/v*) ethanol in a water bath at 80 °C for 30 min with one shaking every 15 min. After adjusting to ambient temperature, ethanol was then added to compensate for the lost weight during the extraction. Finally, the supernatant was filtered through a 0.22 μm membrane filter and injected into the HPLC and LC–MS system for analysis. For animal experiments, the HQT powder was dissolved in water at a temperature of 80 °C and shaken to a final nominal concentration of 0.5 mg dry weight HQT powder extract per mL of water.

The following standard stock solutions were prepared in methanol, stored at 4 °C, and diluted as required for their use in the calibration curves: baicalin (2074.00 mg/L), wogonoside (1200.00 mg/L), oroxylin-A-glucoside (750.00 mg/L), baicalein (270.00 mg/L), wogonin (72.00 mg/L), oroxylin-A (27.20 mg/L), liquiritin (299.00 mg/L), liquiritigenin (129.00 mg/L), isoliquiritoside (6.29 mg/L), isoliquiritigenin (6.43 mg/L), liquiritin apioside (24.90 mg/L) and isoliquiritin apioside (2.94 mg/L).

### 2.3. HPLC and LC–MS analysis

HPLC analysis was performed on a Shimadzu LC-20A system (Kyoto, Japan) equipped with an SPD-20A photodiode array detector. The analytes were separated on a Dikma Diamonsil C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of A (acetonitrile) and B (water containing 1% acetic acid). The linear gradient conditions were optimized as follows: 0–13 min, 17% A; 13–15 min, 17%–20% A; 15–50 min, 20%–30% A; 50–62 min, 30%–40% A; 62–70 min, 40%–44% A; 70–80 min, 44%–100% A. The flow rate was 1.0 mL/min at 35 °C. UV absorbance was monitored at 276 nm for fingerprinting analysis and 276 nm and 370 nm simultaneously for the quantitative analysis.

LC–$MS^n$ analysis was performed on an Aglient Ion Trap6320 mass spectrometer system with an electrospray ion source (ESI) (Applied Biosystems, Toronto, Canada). The LC conditions used for LC–$MS^n$ analysis were the same as those used for the HPLC analysis described above. The optimized MS conditions were fixed as follows: positive ions and negative ions mode was applied. The gas system was set as follows: nebulizer ($N_2$), 42 psi; dry gas ($N_2$), 12 L/min; dry temp., 350 °C. Helium was used as collision gas (compound stability: 100%; trap level: 100%; scan range: 100–1500 Da). The injection volume was 10 μL and all solutions were filtered through a syringe filter (0.22 μm) before analysis.

### 2.4. Method validation

HPLC fingerprinting analysis was developed and its precision, stability and repeatability were validated according to the established guidelines of the China Food and Drug Administration (CFDA) of China[25]. Precision was determined by analyzing the five replicates of a sample of an HQT-S1 solution on the same day. To confirm the repeatability of results, five different sample solutions prepared from the same sample (HQT-S5) were analyzed. Five injections of the same sample HQT-S1 solution at different time points (0, 3, 6, 9 and 12 h) were analyzed to evaluate sample stability. The correlation coefficients between the reference HPLC fingerprints and the sample HPLC fingerprints were calculated as a measure of precision, repeatability and stability.

For quantitative analysis, linearity, lower limit of detection (LLOD) and lower limit of quantification (LLOQ), precision (inter-day and intra-day precision), repeatability, stability, accuracy and specificity were measured and validated according to the guidelines of the International Conference on Harmonization (ICH)[26]. Standard stock solutions containing 11 analytes were prepared and diluted with methanol to the appropriate concentrations to be used in the calibration curves to evaluate LLOD and LLOQ. Calibration curves were generated by plotting peak area against the concentrations of each analyte. The LLOD and LLOQ of the 11 compounds investigated were defined as the amount for which the signal-to-noise ratio (*S/N*) was 3 and 10, respectively. The intra-day and inter-day precisions of the method were assessed by performing 6 replicate injections of a mixed standard solution in day 1 and once daily after that for three consecutive days, respectively. The reproducibility of this method was determined by analyzing 6 independently prepared sample solutions (HQT-S10). The stability was evaluated by repeated analysis of the same sample solution (HQT-S1) every 3 h for a 12 h time period at room temperature. Recovery tests were carried out to determine the accuracy of the method by adding a known amount of the corresponding marker compound to the known sample (HQT-S5). Specificity tests were investigated by using UV spectra and MS fragmentation. The samples were extracted and analyzed as described above. The average percent recoveries were evaluated by calculating the ratio of detected amount *versus* the added amount.

### 2.5. Pharmacological studies

Adult male Wistar rats (190–210 g) were purchased from the Institute of Experimental Animals (Academy of Military Medical Science; Beijing, China), housed under standard conditions (25 ± 5 °C, 40%–70% relative humidity, 12 h/12 h light–dark cycle) and fed the standard diet and water ad libitum. Antipyretic and anti-inflammatory activities were evaluated based on the rat pyrexia models[27] and carrageenin-induced rat hind paws oedema, respectively. Specifically, 1% carrageenin suspension in 0.9% NaCl was injected into the plantar surfaces of the 2 hind paws (0.1 mL/hind paw). Following injection, the volume of the right paw was measured using a YLS-7B Toe Volume Measuring Instrument (Jinan, China). The rectal temperature was measured 3 times daily (at 9:00, 15:00 and 21:00) using a Digital Electronic Thermometer (Shenzhen, China) for 3 days, and only animals with no more than 0.3 °C fluctuations in body temperature were used in the experiments. Sixty rats were randomly assigned into 6 groups of 10 rats each. The following experimental conditions were used. Control group: treated with water; reference group: treated with aspirin (100 mg/kg body weight) dissolved in water, HQT groups: treated with either extract of HQT-S3, HQT-S4, HQT-S6 or HQT-S8 (7.0 g/kg body weight) dissolved in water. In this study, water, aspirin and HQT dosages of 1.4 mL/kg body weight were administrated orally once a day for 3 consecutive days. The last treatment was on day 3, 1 h after the carrageen injection. Response values were recorded at 0, 1, 2, 3, 4, 6 and 8 h as described above. Changes

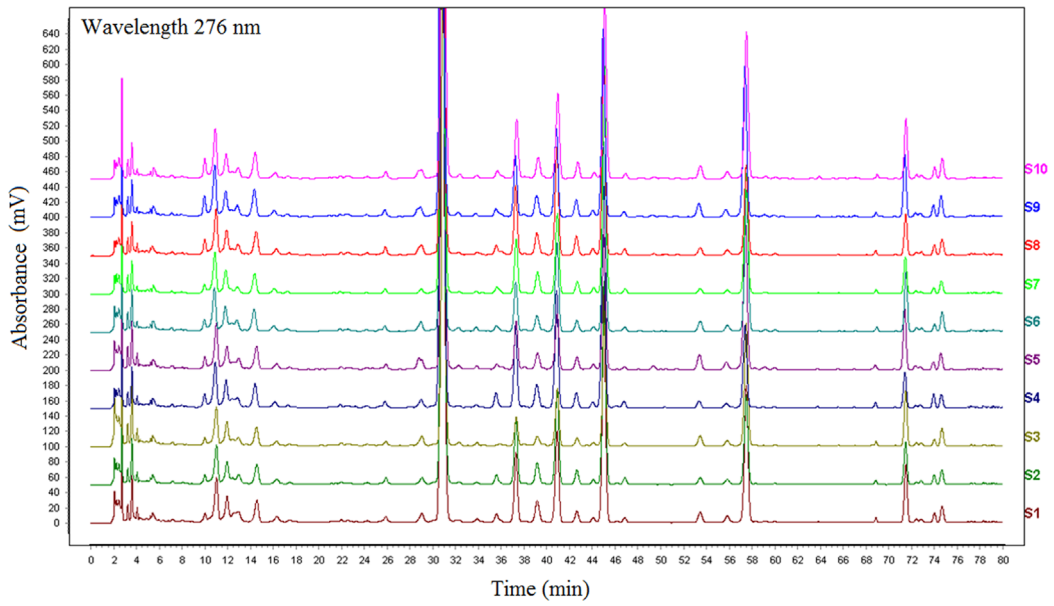



**Figure 1** HPLC fingerprints of 10 batches of Huangqin Tang (similarity 0.990–1.000).

in rectal temperature and paw volume were calculated using the following formula: change in rectal $T$ (°C)$=T_t-T_{0\,h}$; Changes in paw volume (mL)$=(V_t-V_{0\,h})$, where $T_t$ is the rectal temperature at different time points and $V_t$ is the volume of the right paw at different time points. All the rat studies were performed in accordance with the protocols established by the Animal Care and Use Committee of the China Academy of Chinese Medical Sciences.

### 2.6. *Data analysis*

Fingerprinting analysis and quantitative determinations were performed in triplicate. Similarity analysis was performed using the Similarity Evaluation System for Chromatographic Fingerprint of TCM software as established by the Chinese Pharmacopoeia Committee (Version 2008 A). The correlation coefficients of the chromatographic profiles of the samples were calculated and the reference fingerprint chromatogram was calculated and generated.

Principal component analysis (PCA) was used to sort samples into groups[28] using Simca-P11.0 (Umetrics, Umea, Sweden) to differentiate and classify the 10 HQT samples based on the principal peaks. One HQT sample from one group was chosen and evaluated through the proposed pharmacological method. The comparison of mean pharmacodynamic values was done using the Student *t*-test. A *P*-value less than 0.05 was considered significant.

## 3. Results

### 3.1. *Fingerprinting analysis of HQT*

#### 3.1.1. *Method validation*

In order to confirm the accuracy of the equipment, we analyzed 5 successive injections of a sample solution and calculated the correlation coefficients between the reference HPLC fingerprint and the sample HPLC fingerprints. The values obtained were 0.998, indicating that the accuracy of our measurements was satisfactory. Five injections of the same sample solution at different time points (0, 3, 6, 9 and 12 h) were analyzed and the correlation coefficients were calculated to determine whether the sample solutions were stable during the measurement period. The values obtained 0.961 (0 h), 0.961 (3 h), 0.961 (6 h), 0.963 (9 h) and 0.962 (12 h) confirmed that the sample solutions were stable during the measurement periods. At this point, 5 solutions from the same sample were prepared using the same method and were injected individually. The correlation coefficients were 0.999 (0 h), 0.999 (3 h), 0.999 (6 h), 0.999 (9 h), and 0.998 (12 h) demonstrating the repeatability of the analytical method.

#### 3.1.2. *Sample analysis*

Using the parameters described above, we analyzed the 10 batches of HQT samples. The HPLC fingerprinting profiles are shown in Fig. 1. We found 26 common peaks (area greater than 0.5% per chromatogram and common to all chromatograms). Table 1 shows that the similarity between the values obtained for the 10 samples was higher than 0.997, except for HQT-S3 for which the similarity value was 0.990. This indicates a high consistency and stability between the batches of HQT tested.

### 3.2. *Identification of the common peaks*

#### 3.2.1. *Herbal source identification*

The decoction of HQT and its ingredient herbs (namely Radix Scutellariae, Radix et Rhizoma Glycyrrhizae, Radix Paeoniae Alba and Fructus Jujubae) at the concentrations tested were simultaneously analyzed using the chromatographic fingerprinting analysis method. Peaks were assigned to each herb by comparing to the retention time of the peaks in the chromatograms of the individual herbal ingredients. As shown in Fig. 2, 26 peaks in the HQT HPLC fingerprint were unique to an individual herbal component. Specifically, we found the following number of unique peaks: 22 peaks for Radix Scutellariae, 3 peaks for Radix et Rhizoma Glycyrrhizae, 1 peak for Radix Scutellariae and Radix et Rhizoma Glycyrrhizae. These peaks represented 90% of the total area of the chromatogram from 0 to 80 min at a threshold of 0.5%. Small

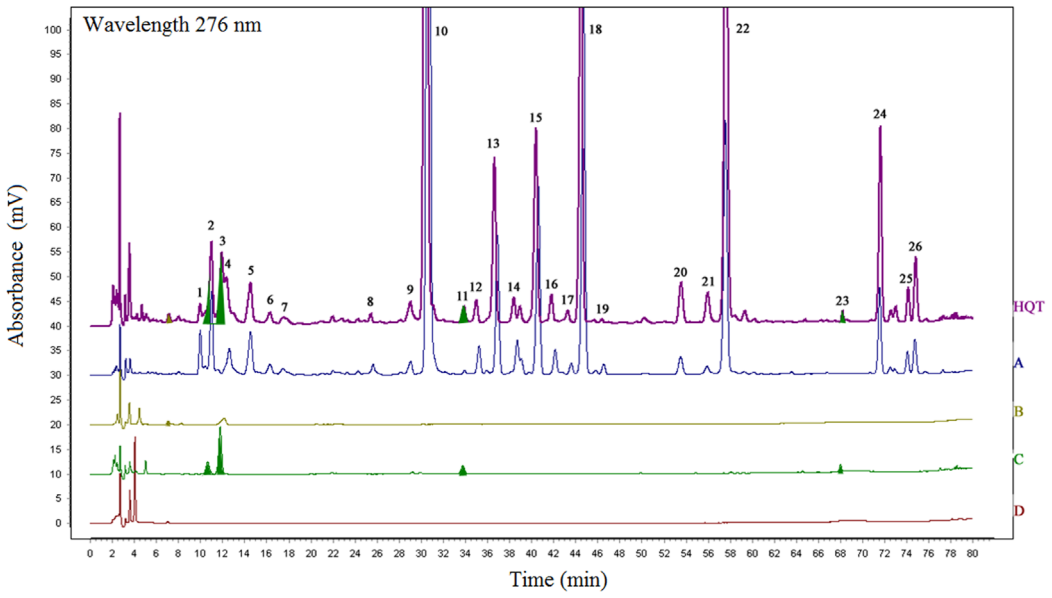



**Figure 2** HPLC chromatograms of Huangqin Tang (HQT) , Radix Scutellariae (A) , Radix Paeoniae (B) , Radix Rhizoma Glycyrrhizae (C) and Fructus Jujubae (D). 26 peaks assigned in the HQT HPLC fingerprint were unique to an individual herbal component; 22 peaks from Radix Scutellariae, 3 peaks from Radix et Rhizoma Glycyrrhizae. 1 common peak is ascribed to Radix Scutellariae and Radix et Rhizoma Glycyrrhizae, simultaneously. These peaks represented 90% of the total area, summed over the overall chromatogram from 0 to 80 min, at a threshold of 0.5%.

peaks in the HQT HPLC fingerprint were ascribed to Radix Paeoniae and Fructus Jujubae.

#### 3.2.2. *Compounds identification*

As shown in Fig. 3 and Table 2, 11 peaks in the HQT HPLC fingerprints (8 peaks in spectra at 276 nm and 3 peaks in spectra at 370 nm) were identified and confirmed using the reference compounds available. An additional 9 peaks were tentatively identified using their UV spectra and MS–MS fragmentation characteristics. The peaks marked with 3, 10, 11, 15, 18, 22, 24 and 26 were liquiritin, baicalin, liquiritigenin, oroxylin-A-glucoside, wogonoside, baicalein, wogonin and oroxylin-A respectively. The peaks marked with 8', 9' and 22' were isoliquiritin apioside, isoliquiritoside and isoliquiritigenin, respectively, which were more readily detected at 370 nm. A list of these identified peaks is provided in Supplementary Fig. 1 and Table 1. No novel compounds were identified. Peak 2 contains liquiritin apioside, a finding that needs to be confirmed in future studies. The 11 confirmed compounds were chosen as markers for quantitative analysis in ten batches of HQT samples.

### 3.3. *Quantitative analysis of HQT*

#### 3.3.1. *Method validation*

All of the peaks in the HPLC profiles obtained from the HQT batches analyzed contained one compound except for peak 2, proving the accuracy of our analysis. However, Peak 3 was only roughly quantified due to limited peak resolution. A detailed description of the calibration curve parameters, precision, reproducibility, stability and recovery is presented in Table 3. Good linearity ($R > 0.999$) was achieved within the investigated ranges for all the analytes tested. The LLOD of the 11 analytes was within the range of 0.034–0.188 mg/L and LLOQ was within the range of 0.068–0.750 mg/L. The results of intra-day and inter-day precisions, their reproducibility and stability showed that the relative standard deviation (RSD) values were all less than 4.0%. The recovery of the method was in the range of 90%–101%, with an RSD less than 4.0% for all analytes. The validation results indicated that the system and the HPLC method were excellent for simultaneous quantitative evaluation of 11 flavonoids in HQT.

#### 3.3.2. *Sample analysis*

The analytical method we developed was successfully applied for the simultaneous determination of 11 compounds in 10 batches of HQT obtained from different sources. Table 4 shows the mean content of each sample, which was analyzed in triplicate and calculated using the external standard method. The distribution of the 11 compounds was very similar among the HQT batches tested. Baicalin was the most common component and isoliquiritigenin was the least common in the batches tested. The relative quantities of the individual components varied with the RSD value. For example, the content of baicalin was in the range of 5.33%–11.10%, with an RSD value of 18.98%. Although Radix et Rhizoma Glycyrrhizae is from the same batch, flavonoids of Radix et Rhizoma Glycyrrhizae showed different distributions in HQT (RSD value, 6.5%–28.0%). The RSD of the total amount of the 11 components was $13.49 \pm 2.13\%$. HQT-S4 contained the greatest abundance of the 11 components identified, specifically baicalin and wogonoside. HQT-S3 contained the lowest abundance, which partly explained the low similarity with the other batches tested. Among the 11 flavonoids identified, baicalin and wogonoside contributed more than 84% of the total composition.

#### 3.3.3. *PCA analysis for the HQT batches*

PCA was performed to distinguish the studied samples using the 11 quantified components identified. The score plots derived from the PCA are shown in Fig. 4. For the following study of animal pharmacodynamic evaluation, the 10 samples could be manually divided into 4 domains based on the score plots. The first domain was HQT-S3 and HQT-S5, and the second one was HQT-S4, which is far from the other samples. The third domain was

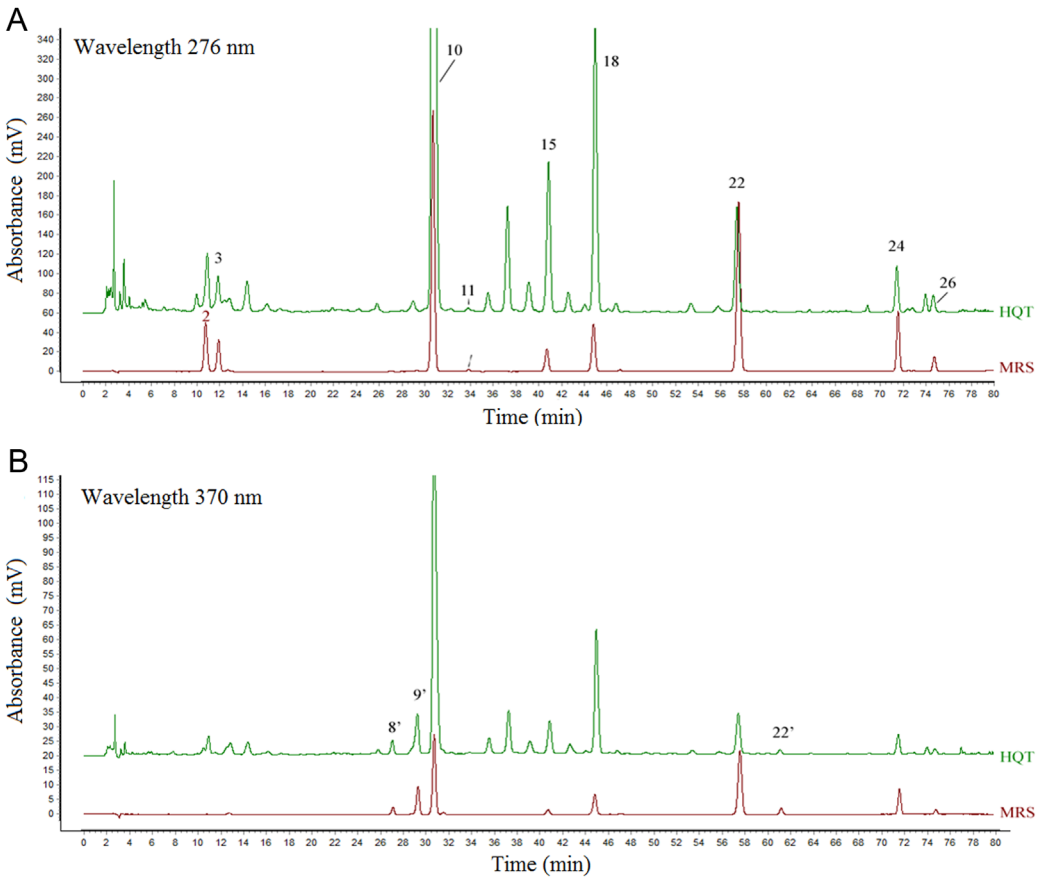



**Figure 3** HPLC profiles of Huangqin Tang (HQT) and mixed reference substances (MRS). 8 peaks of spectra at 276 nm (A) and 3 peaks at 370 nm (B) were confirmed. The peaks marked with 3, 10, 11, 15, 18, 22, 24, 26, 8', 9' and 22' were liquiritin, baicalin, liquiritigenin, oroxylin-A-glucoside, wogonoside, baicalein, wogonin, oroxylin-A, isoliquiritin apioside, isoliquiritoside and isoliquiritigenin. Peak 2 contains liquiritin apioside, which needs to be confirmed in future studies.

**Table 2** LC–MS identification of the common peaks in Huangqin Tang samples.

| Peak No. | Max wave length (nm) | $[M+H]^+$ | Fragmentation ion | Identification |
|---|---|---|---|---|
| 2 | | 550 | 573, 490, 317, 156 | Liquirititin apioside |
| 3 | 280 | 441 | 441, 257 | Liquiritin |
| 8' | 370 | 551 | 573, 515 | Isoliquiritin apioside |
| 9' | 370 | 419 | 419 | Isoliquiritoside |
| 10 | 285, 340 | 469 | 469, 293, 447, 271 | Baicalin |
| 11 | 276 | 257 | 257, 138 | Liquiritigenin |
| 15 | 218, 272, 310 | 483 | 483, 461, 285, 270 | Oroxylin-A-glucoside |
| 18 | 220, 270 | 461 | 461, 285, 270 | Wogonoside |
| 22 | 220, 276, 322 | 271 | 271, 188, 106 | Baicalein |
| 22' | 240, 300, 370 | 257 | 515, 432, 269, 188 | Isoliquiritigenin |
| 24 | 226, 274 | 285 | 285, 270 | Wogonin |
| 26 | 274 | 515 | 514, 432, 269, 285, 270, 203 | Oroxylin-A |

HQT-S1, -S6, -S9 and -S10. The last domain was HQT-S2, -S7 and -S8, which were close to each other.

### 3.4. *Anti-inflammatory and antipyretic activity of HQT*

HQT demonstrated markedly anti-inflammatory, antipyretic, and analgesic efficacy in previous study[1]. Herein, HQT-S3, -S4, -S6 and -S8 were selected for a comparative study of their antipyretic and anti-inflammatory activities. HQT was well-tolerated overall and had no apparent toxic effects. As shown in Fig. 5, HQT-S3, -S4 and -S6 showed statistically significant decreases in fever while HQT-S3, -S4, -S6 and -S8 showed statistically significant decreases in paw edema, compared with the control rats. This study indicates that HQT from different batches has significant antipyretic (all except HQT-S8) and anti-inflammatory effects

($P<0.05$). No significant differences were observed between the different batches of HQT except for the antipyretic effect of HQT-S3 and HQT-S4 at 6 h and the anti-inflammatory effect of HQT-S4, HQT-S6 and HQT-S8. For example, the changes in rectal temperature were $0.64\pm0.46$, $0.80\pm0.42$, $0.61\pm0.22$ and $0.89\pm0.50$ °C and the changes in paw volume were $0.08\pm0.05$, $0.06\pm0.06$, $0.08\pm0.06$ and $0.10\pm0.06$ mL for HQT-S3, -S4, -S6 and -S8, respectively, at 3 h after the last administration of HQT. These *in vivo* results were consistent with the similarities in the chemical fingerprints between these batches.

## 4. Discussion

### 4.1. Optimization of HPLC conditions and extraction methods

Here we investigated the following HPLC conditions: mobile phase, column, detection wavelength and elution program for the optimization of chromatographic conditions. Two different types of LC columns were investigated: Aglient Zorbax SB-C18 (250 mm × 4.6 mm, 5 μm) and Dikma Diamonsil C18 (2) (250 mm × 4.6 mm, 5 μm). The Dikma Diamonsil C18 (2) column was suitable for analysis because it provided better separation efficiency. The HPLC mobile phase in methanol–water, acetonitrile–water and the different concentrations of modifiers (formic acid and acetic acid) were investigated. Finally, acetonitrile–water containing 1% *v/v* acetic acid was applied for the separation of chemical constituents in HQT. The UV detector was employed because of the flavonoid spectra in Radix Scutellariae and Radix et Rhizoma Glycyrrhizae. The monitoring wavelength was set at 276 nm for fingerprinting analysis, whereas 276 nm and 370 nm were simultaneously used for quantitative analysis. Various extraction methods (ultrasonic extraction, cold-dipping under ambient temperature, hot-dipping in an 80 °C water bath), solvents (water, methanol and ethanol at different concentrations), and time were evaluated to obtain the optimized extraction efficiency. According to the total content of the 12 flavonoids, the highest extraction efficiency was obtained with hot-dipping at 75% (*v/v*) ethanol for 30 min in an 80 °C water bath.

### 4.2. Quality control

Bioactive, characteristic, main, synergistic, correlative, toxic and general components are often used as quality control markers of single medicinal herbs[29]. The chemical marker is useful for those herbal medicines of limited and known chemical composition. In addition to the method specified in the *Chinese Pharmacopoeia*, there are many studies on the quality control of Radix Scutellariae, Radix Paeoniae, Radix et Rhizoma and Glycyrrhizaeand Fructus Jujubae[30–33]. In this study, the crude Radix Scutellariae, Radix Paeoniae, Radix et Rhizoma Glycyrrhizae and Fructus Jujubae were collected from their authentic regions, identified by a senior pharmacist according to the requirements of the Beijing Food and Drug Administration. In addition, the content of baicalin in crude Radix Scutellariae was analyzed by HPLC according to the directions of the *Chinese Pharmacopoeia*. However, it is more challenging to ascertain the quality for TCM prescriptions of HQT than for the single medicinal herbs. Unexpected differences were to be found in the compatibility application of the ingredients of HQT after a series of pharmaceutical processing, such as decocting, boiling, concentrating and drying. Chromatographic fingerprinting is a rational and powerful approach to characterize a multi-herb formulation with a complicated chemical profile, and HPLC has proven to be a stable, economical and reliable method for fingerprinting analysis. In this study, 10 batches of crude Radix Scutellariae collected from three different regions and processed according to a standard protocol were analyzed by the HPLC fingerprinting method. Similarity analysis demonstrated that the 10 batches of HQT were homogeneous. Twenty-six common peaks were detected and 11 components were quantified in the chemical profile of HQT. The most common components were the flavonoids from Radix Scutellariae and Radix et Rhizoma Glycyrrhizae (15% of the weight). The distribution of the 11 biologically active components was similar between the different batches. Moreover, our previous studies have shown that 25 (except peak marked 23) of the 26 common peaks in HQT HPLC profiles could enter the body through the intestine and liver in a perfused intestine–liver preparation and most of them demonstrate good pharmacokinetic properties in rats[34,35]. These compounds have applications in the treatment of inflammation, cancer and gastrointestinal disorders. Therefore, HPLC fingerprinting together with simultaneous quantification of the identified flavonoids could be taken into the consideration for quality control of HQT.

Nevertheless, there are still many components that could not be detected due to the limitations of our analysis method. For example, no components were identified for Radix Paeoniae Alba and Fructus Jujubae. Furthermore, the absolutely accurate contents of single or limited constituents maybe not so crucial for the quality control of herbal medicines. There have been some reports about the quality control method using the combination of the chemical profiling and pharmacodynamic measurements[24,36]. In order to provide a complete picture of the composition of HQT, a pharmacological evaluation model was used for full quality assurance. Since an herbal medicine has diverse therapeutic applications, accurate, sensitive and accessible efficacy indicators are critical and necessary for their pharmacological evaluation. HQT possesses a wide range of pharmacological properties such as anti-inflammatory, antipyretic, and analgesic, but in this study we chose not to focus on the latter. Since both fever and paw edema were induced on the carrageenin-induced rats, we established a novel pharmacological model on a single animal for the simultaneous evaluation of the antipyretic and anti-inflammatory effects of HQT samples. Out of the different dosages of HQT tested the dose of 7.0 g/kg body showed the highest efficacy. The PCA results showed that the 4 batches of HQT were chosen for quality assurance. No significant pharmacological differences were observed between the different batches of HQT tested, which indicated consistency in the quality of the different batches, although the component composition varied between the batches. Especially, some batches of HQT are even more effective than aspirin at some points of time. These *in vivo* results supported the findings in chemical fingerprints, suggesting the feasibility and robustness of current strategy for quality control of HQT.

The methodology of fingerprinting qualitative and quantitative analysis provides a strategy for the quality control in TCM. Additionally, an orthogonal pharmacodynamics evaluation would be a useful and efficient complementary quality control method for the herbal medicines, especially for those with unclear effective components. In order to improve the quality control for HQT, future studies should include more batches of HQT from different regions to identify and quantify more constituents and to establish different chromatographic methods. In addition, an evaluation of the pharmacodynamics at the molecular, cellular, organ and

**Table 3** Calibration curve parameters, precision, repeatability, stability and recovery of the 11 compounds in Huangqin Tang.

| Compd. | Regression equation | Linear range (mg/L) | *R* | LLOD (mg/L) | LLOQ (mg/L) | Precision (RSD, %) | | Repeatability (RSD, %) | Stability (RSD, %) | Recovery (%)/RSD (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Intra-day | Inter-day | | | |
| Baicalin | $Y=24{,}378X+495{,}617$ | 11.3–2074.0 | 0.9998 | 0.13 | 0.519 | 2.34 | 0.19 | 1.14 | 1.33 | 95.59/5.03 |
| Wogonoside | $Y=25{,}476X+49{,}692$ | 2.3–1200.0 | 0.9998 | 0.15 | 0.300 | 2.05 | 0.52 | 1.36 | 1.15 | 93.76/4.92 |
| Oroxylin-A-glucoside | $Y=30{,}009X+88{,}403$ | 0.8–750.0 | 0.9998 | 0.188 | 0.750 | 2.20 | 0.37 | 2.3 | 1.63 | 91.43/4.95 |
| Baicalein | $Y=52{,}957X+249{,}968$ | 4.2–270.0 | 0.9992 | 0.14 | 0.281 | 2.38 | 0.37 | 1.07 | 2.27 | 90.08/3.03 |
| Wogonin | $Y=52{,}362X+74{,}302$ | 1.1–72.4 | 0.9992 | 0.065 | 0.129 | 2.45 | 0.18 | 1.22 | 2.4 | 90.09/4.06 |
| Oroxylin-A | $Y=36{,}309X+18{,}262$ | 0.4–27.2 | 0.9992 | 0.034 | 0.068 | 2.26 | 1.11 | 2.33 | 1.54 | 90.00/4.00 |
| Liquiritin | $Y=18{,}488X+12{,}364$ | 4.0–129.4 | 1 | 0.135 | 0.539 | 3.46 | 1.12 | 1.49 | 1.68 | 92.74/3.83 |
| Isoliquiritin apioside | $Y=28{,}043X+1106.9$ | 0.2–6.3 | 0.9999 | 0.131 | 0.524 | 3.56 | 1.54 | 1.6 | 2.40 | 101.23/4.51 |
| Liquiritigenin | $Y=25{,}195X+1247$ | 0.2–6.4 | 0.9999 | 0.134 | 0.268 | 2.39 | 0.87 | 1.64 | 2.50 | 90.03/5.04 |
| Isoliquiritoside | $Y=28{,}572X+4600$ | 0.8–24.9 | 1 | 0.125 | 0.498 | 1.90 | 1.24 | 3.28 | 3.70 | 90.23/4.30 |
| Isoliquiritigenin | $Y=57{,}958X+1310$ | 0.1–2.9 | 1 | 0.049 | 0.196 | 3.10 | 1.35 | 1.22 | 3.90 | 99.07/2.55 |

LLOD, lower limit of determination; LLOQ, lower limit of quantification; *R*, correlation coefficient; RSD, relative standard deviation.

**Table 4** Distribution of the 11 components identified in the batches of Huangqin Tang tested (%) ($n=3$).

| Parameter | Baicalin | Wogonoside | Oroxylin-A-glucoside | Baicalein | Wogonin | Oroxylin-A | Liquiritin | Isoliquiritin apioside | Liquiritigenin | Isoliquiritoside | Isoliquiritigenin | Total contents |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | 10.4149 | 2.4179 | 0.8229 | 0.6754 | 0.2215 | 0.0967 | 0.3843 | 0.0354 | 0.0280 | 0.0865 | 0.0049 | 15.1884 |
| S2 | 9.8605 | 2.2262 | 0.8661 | 0.4274 | 0.1619 | 0.0852 | 0.3195 | 0.0340 | 0.0146 | 0.0769 | 0.0041 | 14.0764 |
| S3 | 5.3292 | 1.3881 | 0.5411 | 0.6409 | 0.2280 | 0.1146 | 0.3721 | 0.0314 | 0.0303 | 0.0783 | 0.0037 | 8.7577 |
| S4 | 11.1016 | 2.4815 | 1.0521 | 0.3804 | 0.1303 | 0.0769 | 0.3951 | 0.0367 | 0.0208 | 0.0885 | 0.0043 | 15.7682 |
| S5 | 7.5986 | 1.5775 | 0.5281 | 0.7885 | 0.2510 | 0.1027 | 0.3559 | 0.0315 | 0.0256 | 0.0707 | 0.0040 | 11.3341 |
| S6 | 8.1003 | 2.0687 | 0.7992 | 0.7126 | 0.2338 | 0.1184 | 0.3426 | 0.0323 | 0.0373 | 0.0736 | 0.0040 | 12.5228 |
| S7 | 10.6043 | 2.6353 | 0.7455 | 0.6102 | 0.1381 | 0.0742 | 0.3464 | 0.0258 | 0.0181 | 0.0656 | 0.0038 | 15.2673 |
| S8 | 10.094 | 2.3343 | 0.9533 | 0.4114 | 0.1498 | 0.0938 | 0.3444 | 0.0361 | 0.0322 | 0.0607 | 0.0035 | 14.5135 |
| S9 | 9.5884 | 2.0784 | 0.7473 | 0.7917 | 0.2396 | 0.1134 | 0.3815 | 0.0330 | 0.0246 | 0.0683 | 0.0036 | 14.0698 |
| S10 | 8.9704 | 2.044 | 0.7966 | 0.7635 | 0.2409 | 0.1238 | 0.3475 | 0.0310 | 0.0222 | 0.0591 | 0.0030 | 13.4020 |
| Min | 5.3292 | 1.3881 | 0.5281 | 0.3804 | 0.1303 | 0.0742 | 0.3195 | 0.0258 | 0.0146 | 0.0591 | 0.0030 | 8.7577 |
| Max | 11.1016 | 2.6353 | 1.0521 | 0.7917 | 0.2510 | 0.1238 | 0.3951 | 0.0367 | 0.0373 | 0.0885 | 0.0049 | 15.7682 |
| Mean | 9.1662 | 2.1252 | 0.7852 | 0.6202 | 0.1995 | 0.1000 | 0.3589 | 0.0327 | 0.0240 | 0.0728 | 0.0039 | 13.4900 |
| RSD, % | 18.9828 | 18.4393 | 20.6454 | 25.7002 | 24.1483 | 17.5545 | 6.5431 | 9.7162 | 28.0428 | 13.6757 | 12.7787 | 15.8116 |

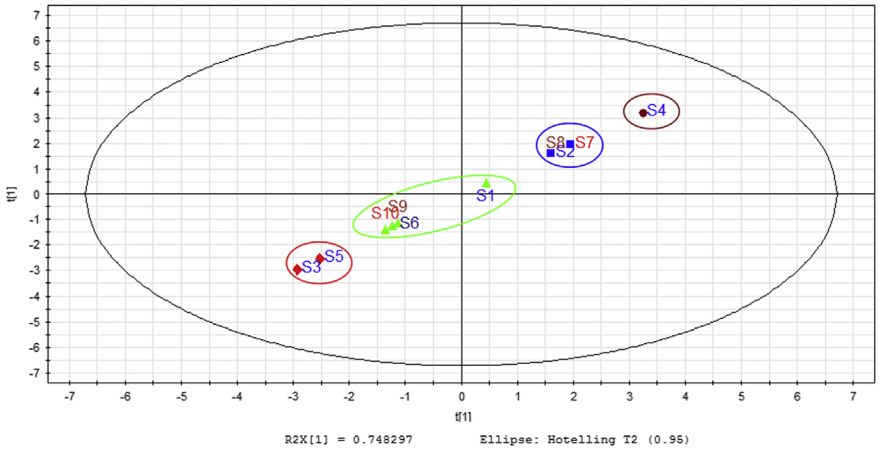



**Figure 4** Analysis of the principal components of the 10 batches of Huangqin Tang based on the input data of the principal components. Ten samples could be manually divided into 4 domains based on the score plots. HQT-S3, -S4, -S6, and -S8 were selected as the markers for the 10 batches for the following pharmacodynamic evaluation.

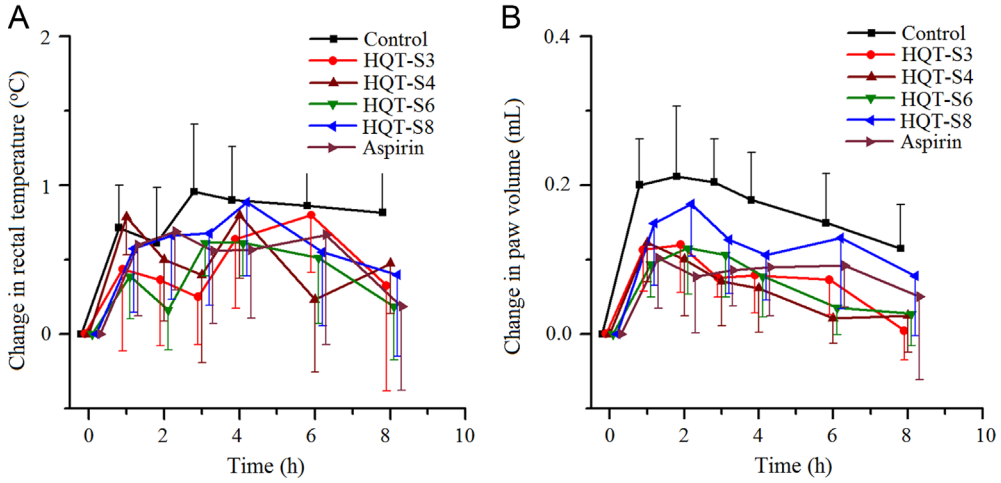



**Figure 5** Antipyretic and anti-inflammatory activities of Huangqin Tang (HQT-S3, -S4, -S6 and -S8) at 7.0 g/kg in carrageenan-induced fever (A) and paw edema (B) in rats ($n=10$).

organism level are needed for a comprehensive and credible evaluation of the compounds.

## 5. Conclusions

In this study, we prepared 10 batches of HQT from different sources with a standard procedure. We detected 26 common peaks in the chemical profile of HQT and ascribed by HPLC. Baicalin, wogonoside, oroxylin-A-glucoside, liquiritin, isoliquiritoside, liquiritin apioside, isoliquiritin apioside and their aglycones were identified and confirmed by LC–$MS^n$. Ten batches of HQT were homogeneous (similarity higher than 0.990). The total contents of flavonoids in the different batches of HQT were consistent. The major components in HQT were baicalin and wogonoside. Finally, small pharmacodynamic differences between different batches of HQT samples were found, demonstrating pharmacological homogeneity of HQT. In conclusion, the results of this study indicate that the combination of chromatographic fingerprint and quantitative analysis could be readily utilized as a quality control approach for the herbal medicines of HQT.

## Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (Nos. 81273662 and 81473592) and the Operational Expenses for Basic Research of China Academy of Chinese Medical Sciences (No. ZZ2014020). The authors would like to thank Dr. Xu Wu and Dr. Jingjing Zhu for their excellent comments and improvement of writing for this manuscript.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2016.01.001.

## References

1. Huang L, Ye WH, Cai BW, Li DF, Liu JF, Liu ML. A preliminary study on the pharmacology of the compound prescription huangqin tang and its component drugs. *China J Chin Mate Med* 1990;**15**:115–7, 28.
2. Zhang BG, Liang XX, Liu QF. Modern pharmacodynamics research and clinic application of huangqintang. *China J Chin Mate Med* 2008;**33**:2587–90.

3. Lam W, Bussom S, Guan F, Jiang Z, Zhang W, Gullen EA, et al. The four-herb Chinese medicine PHY906 reduces chemotherapy-induced gastrointestinal toxicity. *Sci Transl Med* 2010;**2**:45–59.
4. Liu SH, Cheng YC. Old formula, new Rx: the journey of PHY906 as cancer adjuvant therapy. *J Ethnopharmacol* 2012;**140**:614–23.
5. The State Pharmacopoeia Commission of P.R. China. *Pharmacopoeia of the People's Republic of China*. Beijing: China Medicine Science Press; 2010.
6. Ye F, Xui L, Yi J, Zhang W, Zhang DY. Anticancer activity of *Scutellaria baicalensis* and its potential mechanism. *J Altern Complement Med* 2002;**8**:567–72.
7. Kumagai T, Müller CI, Desmond JC, Imai Y, Heber D, Koeffler HP. *Scutellaria baicalensis*, a herbal medicine: anti-proliferative and apoptotic activity against acute lymphocytic leukemia, lymphoma and myeloma cell lines. *Leuk Res* 2007;**31**:523–30.
8. Yoon SB, Lee YJ, Park SK, Kim HC, Bae H, Kim HM, et al. Anti-inflammatory effects of *Scutellaria baicalensis* water extract on LPS-activated RAW 264.7 macrophages. *J Ethnopharmacol* 2009;**125**:286–90.
9. Shang XF, He XR, He XY, Li MX, Zhang RX, Fan PX, et al. The genus *Scutellaria* an ethnopharmacological and phytochemical review. *J Ethnopharmacol* 2010;**128**:279–313.
10. Ikemoto S, Sugimura K, Yoshida N, Yasumoto R, Wada S, Yamamoto K, et al. Antitumor effects of Scutellariae radix and its components baicalein, baicalin and wogonin on bladder cancer cell lines. *Urology* 2000;**55**:951–5.
11. Li-Weber M. New therapeutic aspects of flavones: the anticancer properties of Scutellaria and its main active constituents wogonin, baicalein and baicalin. *Cancer Rreat Rev* 2009;**35**:57–68.
12. Li CR, Lin G, Zuo Z. Pharmacological effects and pharmacokinetics properties of Radix Scutellariae and its bioactive flavones. *Biopharm Drug Dispos* 2011;**32**:427–45.
13. Wu SH, Wu DG, Chen YW. Chemical constituents and bioactivities of plants from the genus *Paeonia*. *Chem Biodivers* 2010;**7**:90–104.
14. Zhang QY, Ye M. Chemical analysis of the Chinese herbal medicine Gan-Cao (licorice). *J Chromatogr A* 2009;**1216**:1954–69.
15. Guo S, Tang YP, Duan JA, Su SL, Qian DW. Chemical constituents from the fruits of *Ziziphus jujuba*. *Chin J Nat Med* 2009;**7**:115–8.
16. He DY, Dai SM. Anti-inflammatory and immunomodulatory effects of *Paeonia lactiflora* Pall., a traditional Chinese herbal medicine. *Front Pharmacol* 2011;**2**:10.
17. Kamei J, Nakamura R, Ichiki H, Kubo M. Antitussive principles of Glycyrrhizae radix, a main component of the Kampo preparations Bakumondo-to (Mai-men-dong-tang). *Eur J Pharmacol* 2003;**469**:159–63.
18. Asl MN, Hosseinzadeh H. Review of pharmacological effects of *Glycyrrhiza* sp. and its bioactive compounds. *Phytother Res* 2008;**22**:709–24.
19. Jiang JG, Huang XJ, Chen J, Lin QS. Comparison of the sedative and hypnotic effects of flavonoids, saponins, and polysaccharides extracted from Semen Ziziphus jujube. *Nat Prod Res* 2007;**21**:310–20.
20. Xie PS, Leung AY. Understanding the traditional aspect of Chinese medicine in order to achieve meaningful quality control of Chinese materia medica. *J Chromatogr A* 2009;**1216**:1933–40.
21. Khan IA. Issues related to botanicals. *Life Sci* 2006;**78**:2033–8.
22. Zhang ZL, Li Q, Li QF, Du SM, Zhou YQ, Lv CM, et al. Simultaneous determination of nineteen major components in Qi She Pill by ultra-high-performance liquid chromatography–tandem mass spectrometry. *Acta Pharm Sin B* 2014;**5**:384–93.
23. Zuo F, Zhou ZM, Liu ML. Determination of 14 chemical constituents in the traditional Chinese medicinal preparation Huangqin-Tang by high performance liquid chromatography. *Biol Pharm Bull* 2001;**24**:693–7.
24. Tilton R, Paiva AA, Guan JQ, Marathe R, Jiang ZL, van Eyndhoven W, et al. A comprehensive platform for quality control of botanical drugs (Phytomics QC): a case study of Huangqin Tang (HQT) and PHY906. *Chin Med* 2010;**5**:30.
25. Drug Administration Bureau of China. Requirements for studying finger print of traditional. *Chinese Medicine Injections (Draft);* 2000.
26. ICH. *Guidance for Industry. Q2 (R1) Validation of analytical procedures: text and methodology*. Geneva: IFPMA; 2005.
27. Autore G, Capasso F, De Fusco R, Fasulo MP, Lembo M, Mascolo N, et al. Antipyretic and antibacterial actions of *Teucrium polium* (L.). *Pharmacol Res Commun* 1984;**16**:21–9.
28. Zhang XH, Shi LF. Studies on different origin of the fruits of *ligustrum lucidum* by chemical pattern recognition. *Pharm J Chin People 'S Lib Army* 2004;**20**:447–9.
29. Liu EH, Qi LW, Li K, Chu C, Li P. Recent advances in quality control of traditional Chinese medicines. *Comb Chem High Throughput Screen* 2010;**13**:869–84.
30. Li CR, Zhou LM, Lin G, Zuo Z. Contents of major bioactive flavones in proprietary traditional Chinese medicine products and reference herb of Radix Scutellariae. *J Pharm Biomed Anal* 2009;**50**:298–306.
31. Li SL, Song JZ, Choi FFK, Qiao CF, Zhou Y, Han QB, et al. Chemical profiling of Radix Paeoniae evaluated by ultra-performance liquid chromatography/photo-diode-array/quadrupole time-of-flight mass spectrometry. *J Pharm Biomed Anal* 2009;**49**:253–66.
32. Sun GX, Wang JQ. Quality control of Radix et rhizoma glycyrrhizae by digitized HPLC fingerprints. *Cent South Pharm* 2009;**7**:51–4.
33. Sun G, Duan JA, Tang YP, Qian YF, Zhao JL, Qian DW, et al. Simultaneous qualitative and quantitative analysis of triterpenic acids, saponins and flavonoids in the leaves of two *Ziziphus* species by HPLC–PDA–MS/ELSD. *J Pharm Biomed Anal* 2011;**56**:264–70.
34. Li T. Study on the chemical basis and pharmacokinetics of Huangqin Tang [dissertation]. Beijing: China Academy of Chinese Medical Sciences; 2013.
35. Li T, Wang YW, Wang YL, Liang RX, Zhang D, Zhang HH, et al. LC–MS quantification and pharmacokinetics of the multi-constituents of Huangqin Tang in rat plasma after different single oral doses. *Acta Pharm Sin* 2013;**48**:917–24.
36. Ren YS, Zhang P, Yan D, Wang JB, Du XX, Xiao XH. A strategy for the detection of quality fluctuation of a Chinese herbal injection based on chemical fingerprinting combined with biological fingerprinting. *J Pharm Biomed Anal* 2011;**56**:436–42.