Comparative Study on “Long-Dan”, “Qin-Jiao” and Their Adulterants by HPLC Analysis

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Abstract “Long-Dan” and “Qin-Jiao” are two important TCM herbs since ancient times in China. In the Chinese Pharmacopoeia, the dried roots and rhizomes of four species from the genus Gentiana, e.g. Gentiana manshurica, G. scabra, G. triflora and G. rigescens, are recorded under the name of Gentianae Radix et Rhizoma (“Long-Dan” in Chinese), while the other four species from the same genus including G. macrophylla, G. crassicaulis, G. straminea and G. duhurica are recorded and used as the raw materials of Gentianae Macrophyllae Radix (“Qin-Jiao” in Chinese). On the basis of the establishment of a validated HPLC–UV method for quantifying simultaneously, five iridoid glycosides, e.g. loganic acid (1), swertiamarin (2), gentiopicroside (3), sweroside (4) and 2’-(o,m-dihydroxybenzyl)sweroside (5) have been used successfully as chemical markers for the comparison of the species used as “Long-Dan”, “Qin-Jiao” and their adulterants in the present study. The results suggested that four iridoid glycosides 1–4 commonly existed in both “Long-Dan” and “Qin-Jiao”, while 2’-(o,m-dihydroxybenzyl)sweroside (5) also existed as one of the major components in “Dian-Long-Dan” species. Moreover, the contents of compounds 1–5 were various in different “Long-Dan” and “Qin-Jiao” species. Herein, we profiled and compared three “Long-Dan” species, four “Qin-Jiao” species and five adulterants by applying multivariate statistical techniques to their HPLC data sets to establish the differences and/or similarities.

Keywords “Long-Dan” · “Qin-Jiao” · Gentiana · HPLC analysis · Iridoid glycosides

1 Introduction

In China, “Long-Dan” is typically used for protecting liver [1], and is commonly used for curing inflammation, hepatitis, rheumatism, cholecystitis and tuberculosis as a well-known traditional Chinese medicinal (TCM) herb [2].

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While, “Qin-Jiao”, another important TCM herb for fighting rheumatism since ancient times in China, has been used as therapy for rheumatism, arthralgia, stroke, hemiplegia, pains, jaundice and infantile malnutrition [3, 4]. The original plants of both “Long-Dan” and “Qin-Jiao” are from the genus Gentiana (Gentianaceae). From which, the dried roots and rhizomes of four species, e.g. Gentiana manshurica, G. scabra, G. triflora and G. rigescens, are recorded under the name of Gentianae Radix et Rhizoma (“Long-Dan” in Chinese) in the Chinese Pharmacopoeia, while the other four species including G. macrophylla, G. crassicaulis, G. straminea and G. duhurica are used as the raw materials of Gentianae Macrophyllae Radix (“Qin-Jiao” in Chinese). In addition to these eight species, most of the Gentiana plants, e.g. G. purdomii, G. microdonta, G. obconica, G. erecto-sepala, G. robusta, have been used as ethno-medicines for “Long-Dan” or “Qin-Jiao” by the local people living in their distributing areas. [5–8].

In general, the qualities and chemical compositions of herbs vary widely, depending substantially on their different species, variety, geographical origin, cultivation, environment, and so on. It was considered that the qualities and chemical compositions of “Long-Dan” and “Qin-Jiao” could be significantly affected by such factors. Different “Long-Dan” and “Qin-Jiao” species previously have been chemically and biologically investigated on by several groups [9–12]. The comparative study on “Long-Dan” and related adulterants by HPLC analysis was also developed by Jiang, et al. [13]. Previous studies suggested that loganic acid, gentiopicroside, sweroside and swertiamarinin, existing widely in genus Gentiana, were the main compounds in “Long-Dan” and “Qin-Jiao”. Among them, loganic acid could inhibit the carrageenan-induced mouse paw edema [14], and gentiopicroside showed inhibitory effects on inflammatory mediators NO and COX-2 [15]. Our recent study showed that iridoid glycosides as the major constituents in “Qin-Jiao” (G. dahurica, G. crassicaulis and G. straminea) and “Long-Dan” (G. rigescens), displayed potential COXs-2/1 inhibitory activities in zebrafish model [12]. However, a detailed comparison among different species used as “Qin-Jiao” and “Long-Dan”, and their related adulterants by applying multivariate statistical techniques is lacking. Herein, a quantitative analysis of five main constituents in Gentiana species, e.g., loganic acid (1), swertiamarinin (2), gentiopicroside (3), sweroside (4) and 2′-(o,m-dihydroxybenzyl)sweroside (5) was established, and their profiling and comparison in 39 Gentiana samples referring to three “Long-Dan” species, four “Qin-Jiao” species and five other relating adulterants were studied by applying multivariate statistical techniques to their HPLC data sets, in order to establish the differences and/or similarities.

Fig. 1 Chemical structures of compounds 1–5

2 Results and Discussion

2.1 Identification of Compounds 1–5

Compounds 1–5 were identified by HPLC–DAD–MS analysis, on the basis of their retention time, UV absorption, the quasi-molecular ions, fragment ions, and co-HPLC comparison with authentic standards, as well as the data published previously. In the LC–MS spectra, the retention times and quasi-molecular ions of the five compounds were as follows: \( t_R = 5.19 \text{ min}, m/z = 375 ([M - H]^-) \) for compound 1; \( t_R = 8.03 \text{ min}, m/z = 397 ([M + Na]^+) \) for compound 2; \( t_R = 10.10 \text{ min}, m/z = 379 ([M + Na]^+) \) for compound 3; \( t_R = 10.70 \text{ min}, m/z = 381 ([M + Na]^+) \) for compound 4; \( t_R = 19.85 \text{ min}, m/z = 493 ([M - H]^-) \) for compound 5.

2.2 Contents of Marker Compounds in Gentiana Samples

The crude methanol extracts of the powdered roots of 39 samples have been prepared, referring to 19 “Long-Dan” samples (S1–S19), seven adulterant samples of “Long-Dan” (S20–S26), 11 “Qin-Jiao” samples (S27–S38) and one adulterant sample of “Qin-Jiao” (S39). The aforementioned samples including 12 different species from 17 different origins were analyzed by HPLC–UV. Table 1 of ESM (S11) listed the concentration of iridoid glycosides identified in “Qin-Jiao”, “Long-Dan” and their adulterants according to species with their relative peak areas (RPA). Five iridoid glycosides were identified as loganic acid (1), swertiamarinin (2), gentiopicroside (3), sweroside (4) and 2′-(o,m-dihydroxybenzyl)sweroside (5) (Fig. 1), through
the comparisons of retention time ($t_R$) and UV absorption with the standards under the same HPLC conditions (Fig. 2). Among them, gentiopicroside (3), one of the main active constituents, was the maximum amount among all the components in both “Long-Dan” and “Qin-Jiao”. The average level of 3 in “Qin-Jiao” (3.51 %) compared with that of them in “Long-Dan” (2.37 %). S19 (G. triflora, collected from Qingyuan, Liaoning) possessed the highest content (4.77 %) of 3 among all the “Long-Dan” samples, while the highest content of 3 (6.30 %) was in S27 (G. crassicaulis, collected from Diqing, Yunnan) among all the “Qin-Jiao” samples.

As for “Long-Dan” samples, the contents of 1–4 in G. scabra and G. triflora were very similar, while those of them in G. rigescens were similar to their adulterants, G. purdumii and G. microdonta (Fig. 3B and SI1). The other two adulterants, G. obconica (S35) and G. erecto-sepala (S24) were not qualified medicinally due to their gentiopicroside (3) content lower than 2 %, according to the record in the Chinese Pharmacopoeia. It is noted that G. rigescens, one of the “Long-Dan” species which is also called “Dian-Long-Dan”, is mainly growing in the southwest of China, particularly in the mountainous areas of Yunnan province [16]. Since compound 5 was only detected in G. rigescens, but not in the other “Long-Dan” species, it could be considered as one of the characteristic components in G. rigescens [17]. Moreover, among the samples of G. rigescens collected from different districts of Yunnan, the content of compound 3 in S7 growing in Kunming area possessed the maximum content (3.50 %), while S9 growing in Lijiang had the lowest content (1.04 %).

Among “Qin-Jiao” and its adulterants, the contents of compounds 1–4 in G. crassicaulis, G. straminea, G. dahurica and G. robusta were quite similar, but higher than those in G. macrophylla (Fig. 3A and SI1). Among them, the total contents of compounds 1 and 3 were less than 2.5 % in two samples, S29 and S30 of G. crassicaulis (collected from Ganzhi in Sichuan provinces, respectively), which could be considered as substandard medicines according to the record in the Chinese Pharmacopoeia. Moreover, the contents of 1 and 3 displayed obviously more different than those of 2 and 4 in different “Qin-Jiao” species (Fig. 3C and SI1).

When comparing of “Long-Dan” with “Qin-Jiao” species, the average contents of compounds 1–4 in “Long-Dan” with 0.38, 0.07, 2.37 and 0.13 %, respectively, were lower than those of them in “Qin-Jiao” with 0.61, 0.20, 3.51 and 0.31 %, respectively. Compound 5 was detected only in one “Long-Dan” species, G. rigescens. The concentrations of compounds 1–5 in different “Long-Dan” species displayed more obviously similar than those of them in different “Qin-Jiao” species (Fig. 3D and SI1).

The aforementioned data showed that the qualities and chemical compositions of herbs depend substantially on their different species, varieties, geographical origins, cultivation, environment, and so on. Furthermore, the contents of marker compounds in three adulterants species, G. purdomii, G. microdonta and G. robusta, were quite similar to the samples of “Long-Dan” and “Qin-Jiao”, respectively.

Fig. 2 HPLC chromatogram of chemical markers 1–5 at 254 nm, and their online UV spectra

![HPLC chromatogram](image-url)
2.3 LC–UV Fingerprint Analysis

Due to the low content of gentiopicroside (3), four samples, G. crassicaulis (S24 and S25 from Ganzi in Sichuan provinces, respectively), G. erecto-sepala (S29), and G. obconica (S30) were not included in the following analysis. As shown in Figs. 4A and 5, seven common peaks were showed up in all the 35 samples. Among which, four peaks were identified as loganic acid (1), swertiamarinin (2), gentiopicroside (3) and sweroside (4), respectively, by comparing of the tR and UV absorption with those of the standard compounds.

In addition to the seven common peaks, two more peaks (tR = 2.37 and 3.18 min) were observed in 24 samples including “Long-Dan” and its adulterants (G. purdomii and G. microdonta) (Fig. 4B). The peak at tR = 21.0 min identified as 2′-(o,m-dihydroxybenzyl)sweroside (5) was observed in all the 16 samples of G. rigescens (S1–S16). The similarity of all the 24 samples of “Long-Dan” and its adulterants was between 0.939 and 0.996. The HPLC fingerprint chromatograms at 254 nm of “Long-Dan” [G. rigescens (S1), G. scabra (S17), G. triflora (S18)], and its adulterants, G. purdomii (S23) and G. microdonta (S26) were shown in Fig. 5. As the major components, loganic acid (1), swertiamarinin (2), gentiopicroside (3), and sweroside (4) were found in all the species. Three characteristic peaks including 2′-(o,m-dihydroxybenzyl)sweroside (5) and peaks d–e were all detected in two “Long-Dan” adulterants, G. purdomii (S20–S23, Fig. 5H) and G. microdonta (S26, Fig. 5I). However, they were not all existed in the other “Long-Dan” samples, suggesting these two adulterants could be distinguished from “Long-Dan” by HPLC analysis.

In the case of 11 “Qin-Jiao” and its adulterants, eight more common peaks (tR = 4.26, 6.67, 8.99, 12.65, 13.27, 14.99, 22.79 and 24.8 min) were observed (Fig. 4C). The similarity indices in 11 samples of “Qin-Jiao” and adulterant samples ranged from 0.960 to 0.999. The HPLC fingerprint chromatograms at 254 nm of “Qin-Jiao” [G. crassicaulis (S27), G. straminea (S31), G. dahurica (S35), G. macrophylla (S38)] and its adulterant [G. robusta (S39)] were shown in Fig. 5. It is noted that peak a showed in all the “Qin-Jiao” samples and its adulterant G. robusta, while not in the “Long-Dan” samples. Although peak a had no
Fig. 4 The chromatographic fingerprints of “Long-Dan”, “Qin-Jiao” and their adulterant samples (A: the total 35 samples; B: “Long-Dan” and its adulterants; C: “Qin-Jiao” and its adulterants; 1: loganic acid; 2: swertiamarinin; 3: gentiopicroside; 4: sweroside; 5: 2’-(o,m-dihydroxybenzyl)sweroside)
identification, the results suggested that peak a was a common typical component in “Qin-Jiao” and “Qin-Jiao” could be distinguished from “Long-Dan” by HPLC analysis on peak a except for four major compounds.

Among the tested samples, *G. purdomii* and *G. microdonta* as the adulterants of “Long-Dan” and *G. robusta* as the adulterant of “Qin-Jiao”, contained all the seven common peaks, accounting for more than 90% of the total peak area. Of them, gentiopicroside (the adulterant of “Qin-Jiao”, contained all the seven Figs. 6, 7 and 8.

According to the fingerprint analysis, seven common compounds, e.g. *G. rigescens* (S7), *G. scabra* (S17), *G. triflora* (S18), *G. crassicaulis* (S27), *G. straminea* (S31), *G. dahurica* (S35), *G. macrophylla* (S38), *G. purdomii* (S23), *G. microdonta* (S26), *G. robusta* (S39); 1: loganic acid; 2: swertiamarinin; 3: gentiopicroside; 4: sweroside; 5: 2’-(o,m-dihydroxybenzyl)sweroside.

2.5 Principal Component Analysis (PCA)

PCA is a kind of a clustering statistical method which reduces the dimensionality of multivariate data to express the original variables as a particular linear combination of the principal components (PCs) in the score plots. Moreover, the plotted data can enhance the visualization of similarities and differences in the data set, allowing for improved discrimination among samples [18, 19]. The relationship of “Long-Dan”, “Qin-Jiao”, and their adulterants from 10 *Gentiana* species was investigated on by PCA using the data of seven common peaks 1–7. As shown in Fig. 9. Ten *Gentiana* species could be clearly discriminated in the score plots constructed by combining PC 1 (41.5%) and PC 2 (23.2%). From the score plots, most of the “Qin-Jiao” and “Long-Dan” species were separated by PC1 whereas some samples from “Long-Dan” species, e.g. S17 (G. scabra) and S18-S19 (G. triflora) were in the area of “Qin-Jiao”. The result indicated that “Long-Dan” and “Qin-Jiao” could not be discriminated from each other by using these seven common peaks in the present study. And this might be the reason that “Long-Dan”, “Qin-Jiao” and their adulterants have been easily confused by the local people. From the phytochemical point of view, it is important to increase the characteristic components of “Long-Dan” and “Qin-Jiao”, in order to distinguish them reasonably.

3 Experimental

3.1 General

Loganic acid (1) and 2’-(o,m-dihydroxybenzyl)sweroside (5) were isolated by our laboratory and confirmed by NMR and MS spectroscopy for structures [20, 21] and HPLC for purity (>98%). Swertiamarinin (2), gentiopicroside (3), and sweroside (4) were bought from the National Institute for the Control of Pharmaceutical and Biological (NIC-PBP). MeOH (chromatographic grade), acetonitrile (chromatographic grade) and phosphoric acid (reagent grade) were purchased from Merck (Darmstadt, Germany). Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA). RC membrane filters, 0.45 μm, Φ 25 mm, were purchased from IVA (Meerbusch, Germany).
Dendrogram using Average Linkage (Between Groups)

**Fig. 6** Dendrogram of clustering analysis for “Long-Dan” and its adulterant. (24 samples)

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Dendrogram using Average Linkage (Between Groups)

Rescaled Distance Cluster Combine

| CASE | Label | Num |
|------|-------|-----|
|      |       | 0   |
|      |       | 5   |
|      |       | 10  |
|      |       | 15  |
|      |       | 20  |
|      |       | 25  |
| S1   | 1     |
| S12  | 12    |
| S13  | 13    |
| S7   | 7     |
| S4   | 4     |
| S16  | 16    |
| S17  | 17    |
| S18  | 18    |
| S19  | 19    |
| S20  | 20    |
| S23  | 23    |
| S21  | 21    |
| S22  | 22    |
| S8   | 8     |
| S10  | 10    |
| S9   | 9     |
| S3   | 3     |
| S5   | 5     |
| S26  | 24    |
| S2   | 2     |
| S11  | 11    |
| S14  | 14    |
| S15  | 15    |
| S6   | 6     |
```

Dendrogram using Average Linkage (Between Groups)

**Fig. 7** Dendrogram of clustering analysis for “Qin-Jiao” and its adulterant. (11 samples)
### 3.2 Plant Material

The studied plant materials (Table 1 of ESM) included 26 “Long-Dan” samples from three officinal species of *G. rigescens* (S1–S16), *G. scabra* (S17), *G. triflora* (S18–S19), and four adulterants including *G. purdomii* (S20–S23), *G. erecto-sepala* (S24), *G. obconica* (S25) and *G. microdonta* (S26), and 13 “Qin-Jiao” samples from four officinal species, e.g. *G. crassicaulis* (S27–S30), *G. straminea* (S31–S34), *G. dahurica* (S35–S37) and *G. macrophylla* (S38), and one related adulterant, *G. robusta* (S39). Since *G. manshurica*, one of the “Long-Dan” officinal species is tending to extinguish and hard for collecting in the open field, it is lacking in the sample list.

The samples were collected in southwestern China (Yunnan and Sichuan provinces) for *G. rigescens*, *G. purdomii*, *G. crassicaulis*, and *G. macrophylla*, in northeastern China (Jilin and Liaoning provinces) for *G. scabra* and *G. triflora*, in southwestern and northwestern China (Tibet, Qinghai and Gansu provinces) for *G. straminea*, *G. dahurica* and one related adulterant, *G. robusta*.
dahurica, and G. macrophylla, and in Tibet for G. robusta, G. erecto-sepala, and G. obconica, respectively. All of the plant materials were collected from February to June of 2011. The botanical origins of all the collected samples were identified by Dr. Shu-Dong Zhang and Rong Li from Kunming Institute of Botany (KIB), Chinese Academy of Sciences (CAS), during the field collection. The specimens of all these materials were deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, KIB, CAS. The voucher numbers were shown in Table 1 of ESM (SI1).

3.3 HPLC and HPLC–MS Analysis

The powdered roots (0.25 g) of each sample were immersed in MeOH (10 mL) over eight hours and then extracted under ultrasonic condition for 30 min. The obtained residue was filtered through a syringe filter (0.45 μm), and an aliquot of each filtrate (10 μL) was injected into the HPLC instrument for analysis. HPLC analysis was performed on an Agilent series 1260 (Agilent Technologies) liquid chromatography, equipped with a vacuum degasser, a quaternary pump, an autosampler, and a diode array detector (DAD). An Agilent ZORBAX SB-C18 column (4.6 × 150 mm, 5 μm) was used. The following gradient system was used with water containing 0.2 % (v/v) H₃PO₄ (solvent A) and acetonitrile (solvent B): 0–25 min: linear 8–20 % of B; 25–26 min: linear 20–100 % of B. The flowing rate was 1 mL/min, and the detection wavelength was at 254 nm. Diode array detection was between 190 and 650 nm and the column temperature was set at 40 °C and the monitored wavelength was 254 nm.

HPLC–DAD–MS analysis was performed on an Agilent series 1100 (Agilent Technologies) liquid chromatography, equipped with a vacuum degasser, a quaternary pump, an autosampler, and a DAD and an ion-trap mass spectrometer with electrospray interface (ESI), operating in full scan MS mode from 150 to 1,500 amu. Samples were analyzed using both negative and positive ionization modes. ESI–MS parameters were as follows: potential of the ESI source, 4 kV; capillary temperature, 400 °C. An Agilent ZORBAX SB-C₁₈ column (4.6 × 150 mm, 5 μm) was used. The mass traces of five were recorded, and identification of individual compounds was conducted by MSⁿ fragmentation and comparison with standards. The gradient system was the same system as described in the above HPLC conditions part. HPLC injection volume was 10 μL. The result was shown in Figs. 2 and 3 of ESM (SI5 and SI6).
3.4 Calibration of Compounds 1–5

Standard samples of compounds 1–5 were prepared into appropriate concentration, and the calibration curve for each compound was performed with six different added quantities in triplicate by plotting the peak area versus the quantities of the compounds. All five calibration curves exhibited good linear regressions, and the results are shown in Table 2 and Fig. 1 of ESM (SI2 and SI3).

3.5 Method Evaluation

Selectivity was determined by comparing the chromatograms obtained from the Gentiana samples with those of the standard solutions. Precision was calculated in terms of intra-day (n = 6) with the standard solution of compounds 1–5 on the Agilent ZORBAX SB-C18 column and evaluated by calculating the relative standard deviation (RSD). In order to test the repeatability, solutions of sample 1 were prepared and it was injected 6 times (Table 3 of ESM, SI4). Other method evaluation was performed as described by our previous studies [22].

3.6 Data Analysis

A professional and recommended software by the SFDA of China, named Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004 A) was used for similarity analysis of chromatographic profiles of “Long-Dan”, “Qin-Jiao” and their adulterants. By which, seven common peaks in the chromatograms were selected and the peak of gentiopicroside (3) was used as the reference. The relative retention time (RRT) and RPA of each common peak to the reference in the chromatograms were calculated. The hierarchical clustering analysis (HCA) of 35 samples was performed with between-group linkage method in SPSS (version 16.0, USA). In addition, principal component analysis (PCA) was also applied to clarify the relationship between these species by using SIMCA-P (version 11.0 Umetrics, Umea, Sweden).

4 Conclusions

A validated HPLC–UV method for simultaneously quantifying of five iridoid glycosides, e.g. loganic acid (1), swertiamarinin (2), gentiopicroside (3), sweroside (4) and 2′-(o,m-dihydroxybenzyl)sweroside (5) in “Long-Dan”, “Qin-Jiao” and their adulterants was established in the present study. It was found that the chemical constituents of “Long-Dan”, “Qin-Jiao” and their adulterants were differed from each other, even among the samples from the same species, due to different geographical positions and climatic conditions, which may cause the qualitative differences between the plants from various areas.

In the Chinese Pharmacopoeia, it recorded that the content of gentiopicroside (3) should be no less than 2 % in “Long-Dan” with an exception for G. rigescens (no less than 1 %), and the total contents of gentiopicroside (3) and loganic acid (1) must be no less than 2.5 % in “Qin-Jiao”.

Conclusions

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Conflict of interest The authors declare no conflict of interest.

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