

# Chemical Characteristics Combined with Bioactivity for Comprehensive Evaluation of Tumuxiang Based on HPLC-DAD and Multivariate Statistical Methods

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## ABSTRACT

**Background:** The dried roots of *Inula helenium* L. (IH) and *Inula racemosa* Hook f. (IR) are used commonly as folk medicine under the name of “tumuxiang (TMX)”. Phenolic acid compounds and their derivatives, as main active constituents in IH and IR, exhibit prominent anti-inflammation effect.

**Objective:** To develop a holistic method based on chemical characteristic and anti-inflammation effect for systematically evaluating the quality of twenty-seven TMX samples (including 18 IH samples and 9 IR samples) from different origins.

**Methods:** HPLC fingerprints data of AL (*Aucklandia lappa* Decne.) whose dried root was similar with HR was added for classification analysis. The HPLC fingerprints of twenty-seven TMX samples and four AL samples were evaluated using hierarchical clustering analysis (HCA) and principle component analysis (PCA). The spectrum-efficacy model between HPLC fingerprints and anti-inflammatory activities was investigated by principal component regression (PCR) and partial least squares (PLS).

**Results:** All samples were successfully divided into three main clusters and peaks 7, 9, 11, 22, 24 and 26 had a primary contribution to classify these medicinal herbs. The results were in accord with the appraisal results of herbs. The spectrum-efficacy relationship results indicated that citric acid, quinic acid, caffeic acid- $\beta$ -D-glucopyranoside, chlorogenic acid, caffeic acid, 1,3-O-dicaffeoyl quinic acid, tianshic acid and 3 $\beta$ -Hydroxypterondontic acid had main contribution to anti-inflammatory activities.

**Conclusion:** This comprehensive strategy was successfully used for identification of IH, IR and AL, which provided a reliable and adequate theoretical basis for the bioactivity relevant quality standards and studying the material basis of anti-inflammatory effect of TMX.

**Key words:** *Inula helenium* L., *Inula racemosa* Hook f., HPLC fingerprints, Spectrum-efficacy relationship, Multivariate statistical methods

**Abbreviations:** IH: *Inula helenium* L.; IR: *Inula racemosa* Hook f.; TMX: Tumuxiang; AL: *Aucklandia lappa* Decne.; HCA: Hierarchical clustering analysis; PCA: Principle component analysis; PCR: Principal component regression; PLS: Partial least squares; TCMs: Traditional Chinese Medicines; ChP 2015: Chinese Pharmacopoeia 2015; WHO: World health organization; FDA: Food and drug administration; HPLC-DAD: High-performance liquid chromatography-diode array detection; GC-MS: Gas chromatography-mass spectroscopy; HPLC-MS: High-performance liquid chromatography-mass spectroscopy; UPLC-MS: Ultra high-performance liquid chromatography-mass spectroscopy; RMSE: Root mean square error; RMSECV: Root mean square error of cross-validation; RRT: Relative retention time; RPA: Relative peak area; RSDs: Relative standard deviations

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## INTRODUCTION

There are more than 20 species of the genus *Inula* widely found in China<sup>[1]</sup>. Among of them, the root of *Inula racemosa* Hook f. (IR) and *Inula helenium* L. (IH) are used commonly as folk medicine under the name of “tumuxiang (TMX)”, and the later is especially used by Tibetan and also named “Manu”<sup>[2]</sup>. IH is recorded in Chinese Pharmacopoeia 2015 (ChP 2015) while IR is not recorded<sup>[3]</sup>. Since IH and IR possess anti-inflammatory, fungicidal, anticancer properties and other biological activities<sup>[4-7]</sup>, they have been widely used for the treatment of abdominal distension and pain, acute enteritis and bacillary dysentery<sup>[8-9]</sup>. The studies on the chemical composition of IH and IR showed that both of them contained the active ingredients alantolactone, isovalantolactone, dihydroalantolactone and dihydroisovalantolactone<sup>[10-11]</sup>. However, IH also

contained  $\beta$ -elemene, alantol, inulic acid and aromadendrene<sup>[12-13]</sup>, which were not contained in IR. IR contained sitisterol, daucosterol, inunolide, apotaxene, phenylacetone and isoinulal<sup>[14]</sup>, while these chemical constituents were not found in IH. The dried root of IH and IR are difficult to distinguish because of the similarity of their morphology. In fact, it's a universal phenomenon that IH and IR are easily confused in the process of medicinal materials circulation and clinical use<sup>[2]</sup>. Because of the chemical composition of IH and IR is obvious difference, whether they can be substituted each other has been a problem urgently to be solved by pharmaceutical workers. Aiming at this problem, first of all, it's in need of developing a rapid and effective identification method for two herbs.

The chromatographic fingerprinting has been widely used for the identification, authentication, characterization and classification of herbal medicines<sup>[15-16]</sup> and it is accepted and

advised as an efficient method to control the quality of herbal medicines by world health organization (WHO) and food and drug administration (FDA)<sup>[17–18]</sup>. Modern hyphenated chromatographic techniques, such as high-performance liquid chromatography-diode array detection (HPLC-DAD), gas chromatography-mass spectroscopy (GC-MS) and high-performance liquid chromatography-mass spectroscopy (HPLC-MS), ultra high-performance liquid chromatography-mass spectroscopy (UPLC-MS), have become the most frequently applied and powerful techniques in analyzing such complicated systems. HPLC fingerprint analysis has been drawn much attention owing to its stable performance and getting large amount of information<sup>[19]</sup>. Multivariate statistical methods, HCA (hierarchical cluster analysis), PCA (principal component analysis), PCR (principal component regression) and PLS (partial least square) are frequently applied to analysis chromatographic fingerprints of TCMs to achieve the classification and identification of TCMs<sup>[20]</sup>.

It has been widely acknowledged that TCMs take effects via a holistic mode of multiple-components and multiple-targets, the conventional modes based on the quantitative analysis of single active ingredient or several index ingredients are not appropriate for reflecting the holistic bioactivity of TCMs. Meanwhile, the selection of index ingredients usually lack sufficient chemical and pharmacological evidence, resulting in poor bioactivity relevance in the quality control<sup>[21]</sup>. Spectrum-efficacy relationship is an emerging approach to facilitating the bioactivity relevant quality control of TCMs<sup>[22–23]</sup>. There is no doubt that fingerprint-activity approach fits very well with the holistic mode of TCMs eliciting the pharmacological efficacy via multiple components. Liu et al. investigated the spectrum-effect relationship between UPLC fingerprints and energy metabolism of mitochondria of Fuzi, Yanfuzi, Heishunpian and Paofupian by using canonical correlation analysis, the results showed a representative way to discover the primary active ingredients from the complicated herbal drugs<sup>[24]</sup>. Shan et al. had reported that the spectrum-efficacy model between HPLC fingerprints and T-lymphocyte proliferative activities of *Panax ginseng* C.A. Meyer was investigated by PCR and PLS, that revealed the real bioactive compounds acting on the lymphocyte proliferative capacity<sup>[25]</sup>.

In this study, the chemical fingerprint characteristics of IH and IR in twenty-seven samples from four different geographical regions were analyzed by HPLC-DAD, for studying the differences between IH and IR from the perspective of the overall chemical compositions. In order to verify whether the taxonomic methods could be successfully applied to the classification and identification of the easily confused herbs, HPLC fingerprints data of AL whose dried root was similar with HR was added for HCA and PCA. Hence, HCA and PCA were performed to evaluate the HPLC fingerprints of twenty-seven TMX samples and four AL samples. The anti-inflammatory capacity of the 27 TMX samples (S1-27) was studied by using the inflammatory model of auricle edema induced by dimethylbenzene in mice. The spectrum-efficacy relationship of HPLC fingerprints and anti-inflammatory

activities was established by multivariate calibration techniques to simultaneously assess the predominant active components. The structure identification of the detected characteristic peaks was achieved by comparing their resolved spectra with the standard ones as well as the MS/MS fragments. Figure 1 displayed the entire analytical process of this work. On the one hand, the proposed strategy provided a method for the chemical identification of IH and IR; on the other hand, it could discover anti-inflammatory activity ingredients and provided the basis to select quality control indexes of TMX associated with anti-inflammatory activities.

## METHODS

### 1. Materials and reagents

HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Distilled water was further purified using a Milli-Q system (Millipore, Milford, MA, USA). Analytical grade formic acid was obtained from Nanjing Chemical Reagent CO. LTD. Citric acid, quinic acid, caffeic acid- $\beta$ -D-glucopyranoside, chlorogenic acid, caffeic acid, 1,3-O-dicaffeoyl quinic acid, tianshic acid, 3 $\beta$ -Hydroxypterondontic acid were purchased from Shanghai Research Center of Chinese Medicine Standardization. All solvents were filtered through a 0.45  $\mu$ m filter before injecting into HPLC.

Both TMX and AL grew at an altitude of 2000 meters above. Twenty-seven TMX samples and four AL samples were collected from the provinces of Gansu, Qinghai, Sichuan, Tibet and Yunnan during two years (Table 1). The voucher specimens were identified by Dr.Yinsuo Zhou (Lanzhou University).

### 2. Sample preparation

1.0 g of TMX powder after passing through a 40 mesh sieve was weighed accurately and introduced into a 50 mL round-bottom flask, and 20 mL water was added. The mixture was reflux extracted for 2 h and then allowed to stand for 60 min at room temperature to cool down. The extraction solution was filtered through a 0.45  $\mu$ m syringe filter prior to HPLC analysis. AL samples were prepared with the same procedure.

### 3. Instrumentation and chromatographic condition

#### 3.1. Liquid chromatography

A waters e2695 separations module equipped with a 1250 binary pump, a 2998 diode array detector (DAD) and a 717 plus autosampler was utilized to obtain HPLC fingerprints chromatograms. The system was controlled by Empower 3 software (Waters, USA). The chromatographic separation was performed using a Diamonsil C<sub>18</sub> (250 $\times$ 4.6 mm, 5  $\mu$ m) maintained at 30 °C. The mobile phase was acetonitrile (A) and 0.1% formic acid water (B) with a gradient program as follows: 0–8 min, 5% A, 8–25 min, 5–15% A, 25–40 min, 15–23% A, 40–45 min, 23–30% A, 45–55 min, 30–60% A, 55–65 min, 60–100% A, 65–70 min, 100% A. Other chromatographic parameters were as follows: the flow rate was 1 mL/min, the wavelength of detector was set at 280 nm. All samples were injected with 20  $\mu$ L.

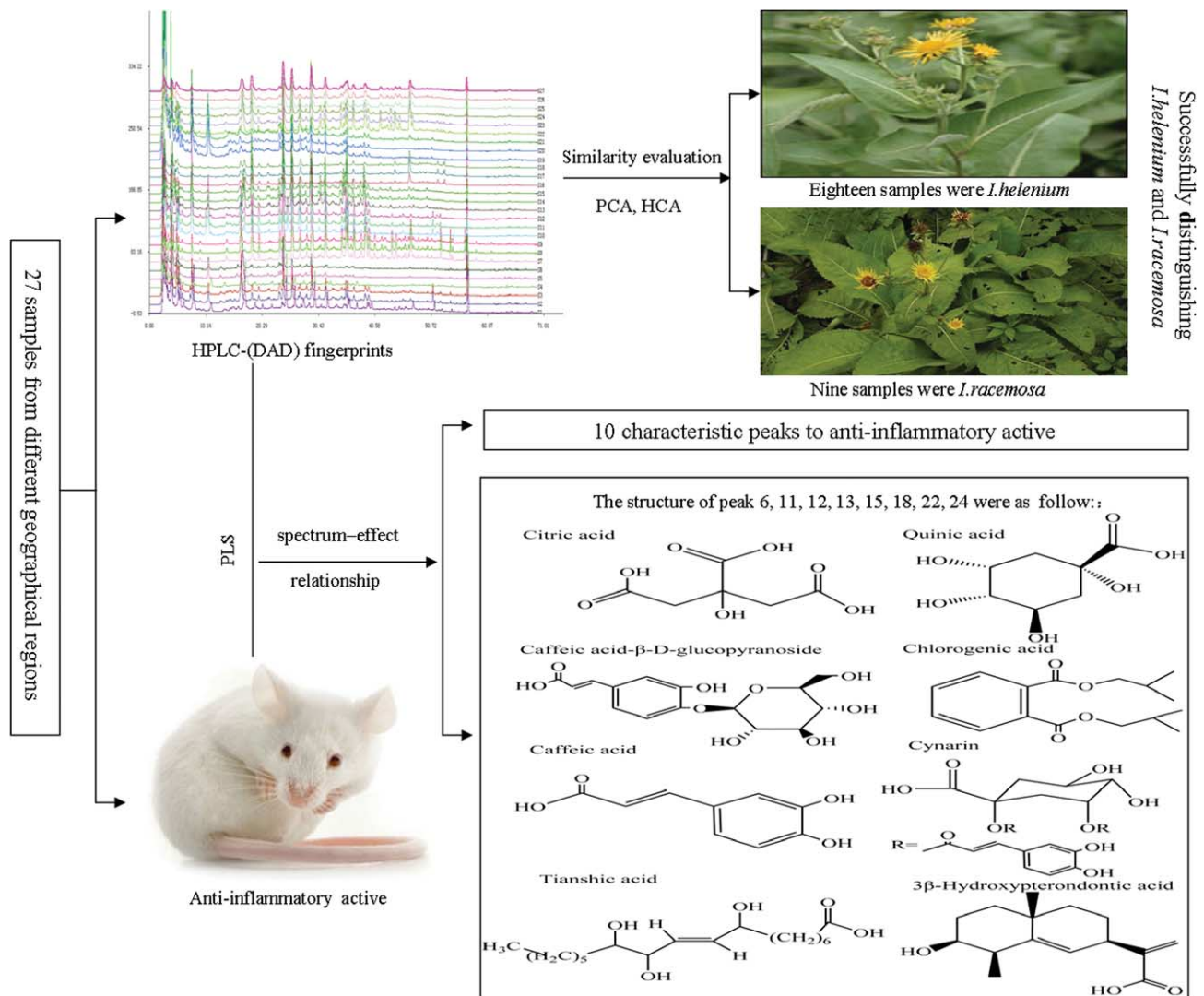


Figure 1. The strategy for identifying IH, IR and revealing their anti-inflammatory active ingredients.

Table 1. A summary of tested samples

No.	Name	Region	Collection date	No.	Name	Region	Collection date
S1	IHQHXN1	Xining, Qinghai	2013.06.08	S16	IHTZSMZ1	Shimen zheng,Ganshu	2014.7.22
S2	IHQHXN2	Xining, Qinghai	2013.06.08	S17	IHTZSMZ2	Shimen zheng,Ganshu	2014.7.22
S3	IHQHXN3	Xining, Qinghai	2013.06.08	S18	IHTZSMZ3	Shimen zheng,Ganshu	2014.7.22
S4	IHQHXN4	Xining, Qinghai	2013.08.20	S19	IHSCCD1	Chendu, Sichuan	2013.7.8
S5	IHQHXN5	Xining, Qinghai	2013.08.20	S20	IHSCCD2	Chendu, Sichuan	2013.7.8
S6	IHQHXN6	Xining, Qinghai	2013.08.20	S21	IHSCCD3	Chendu, Sichuan	2013.7.8
S7	IRQHKM1	Kumbum Monastery,Qinghai	2013.3.9	S22	IRGSGN1	Gannan, Ganshu	2013.8.20
S8	IRQHKM2	Kumbum Monastery,Qinghai	2013.3.9	S23	IRGSGN2	Gannan, Ganshu	2013.8.20
S9	IRQHKM3	Kumbum Monastery,Qinghai	2013.3.9	S24	IRGSGN3	Gannan, Ganshu	2013.8.20
S10	IHQHKM4	Kumbum Monastery, Qinghai	2013.7.15	S25	IRTCD1	Changdu, Tibet	2013.8.20
S11	IHQHKM5	Kumbum Monastery, Qinghai	2013.7.15	S26	IRTCD2	Changdu, Tibet	2013.8.20
S12	IHQHKM6	Kumbum Monastery, Qinghai	2013.7.15	S27	IRTCD3	Changdu, Tibet	2013.8.20
S13	IHGSTZF1	Tianzhu,Ganshu	2014.7.22	S28	ALSCCD1	Chendu, Sichuan	2013.9.13
S14	IHGSTZF2	Tianzhu,Ganshu	2014.7.22	S29	ALSCCD2	Chendu, Sichuan	2013.9.11
S15	IHGSTZF3	Tianzhu,Ganshu	2014.7.22	S30	ALSCCD1	Yunnan	2013.9.9
				S31	ALSCCD2	Yunnan	2013.9.14

Note: *Inula helenium* L. (abbreviated: IH) and *Inula racemosa* Hook f. (abbreviated: IR) and *Aucklandia lappa* Decne. (abbreviated: AL). Principles of naming: species abbreviations add the initial letter of collection areas.

### 3.2. Mass spectrometry

All experiments were conducted with a Quattro Premier XE Micro mass spectrometer (Waters Corporation) equipped with an electrospray ionization (ESI) source interfaced with an HPLC system (Waters Corporation). MS detection was performed in both positive ion and negative ion mode. The source parameters were electrospray capillary voltage 3.0 kV for positive ionization mode and 2.8 kV for negative ionization mode, source temperature 120 °C and desolvation temperature 300 °C. The cone voltage of 40 V was used throughout, with extractor voltage of 3 V and the RF lens voltage was set at 0.1 V. The desolvation and cone gas flows were set at 600 h/L and 50 h/L, respectively.

### 4. Animals

Kunming mice (18–22 g body weight, n=10 per group) were obtained from the Laboratory Animal Center of Lanzhou University (Lanzhou, China) and housed with unlimited access to food and water, the animals were maintained on an ambient temperature of 22–25 °C and a relative humidity of 60%. All animals were housed for 1 week before experiment. All animal experiments were conducted in accordance with the Guidelines for Animal Experimentation of Lanzhou University, and the protocol was approved by the Animal Ethics Committee of that institution.

### 5. Anti-inflammatory testing

The anti-inflammatory property of TMX was investigated using the model of auricle edema induced by dimethylbenzene in mice. The mice were divided into twenty-nine groups of 10 each. The positive group, test groups and blank group were intragastric administered with reference drug (indomethacin, 30 mg/kg), tested extracts (9 g/kg) and normal saline for 10 days respectively. On the tenth day after administration, the mice were topical applied 0.05 mL dimethylbenzene to both inner and outer surface of right ear. Mice were sacrificed by cervical dislocation 15 min later, and then the ear biopsies of both ears were obtained with a punch (a diameter of 8 mm) and weighed. Weight-increase-rate of the right ear over the left one indicated the vasodilatation<sup>[26]</sup>.

### 6. Data analysis

#### 6.1. Data pretreatment

The data set consisted of an n×m matrix, in which rows represented the 27 TMX samples and 4 AL samples, and columns for the 28 peaks area for all data analyses. The similarity analysis was performed using Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004 A). HCA, PCA and PCR were implemented using SPSS 18.0 software. PLS was performed by SIMCA-P 11.5 Demo for statistical analysis.

#### 6.2. Similarity analysis

The similarity analysis of IH and IR samples was carried out to determine the degree of similarity or dissimilarity of samples from each other. HPLC-DAD chromatogram data of all samples was submitted for analysis by the professional

software of Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A). Then, S1 was chose as the reference chromatogram and the peaks would be matched. Subsequently, the standard chromatogram was produced, and the similarity value of all input chromatograms relative to standard chromatogram would be calculated by using the cosine value of the angle<sup>[27]</sup>. They were formulated respectively as follows<sup>[28]</sup>:

$$R_{\text{con}} = \frac{\sum_{i=1}^n X_i Y_i}{\sqrt{\sum_{i=1}^n X_i^2} \sqrt{\sum_{i=1}^n Y_i^2}}$$

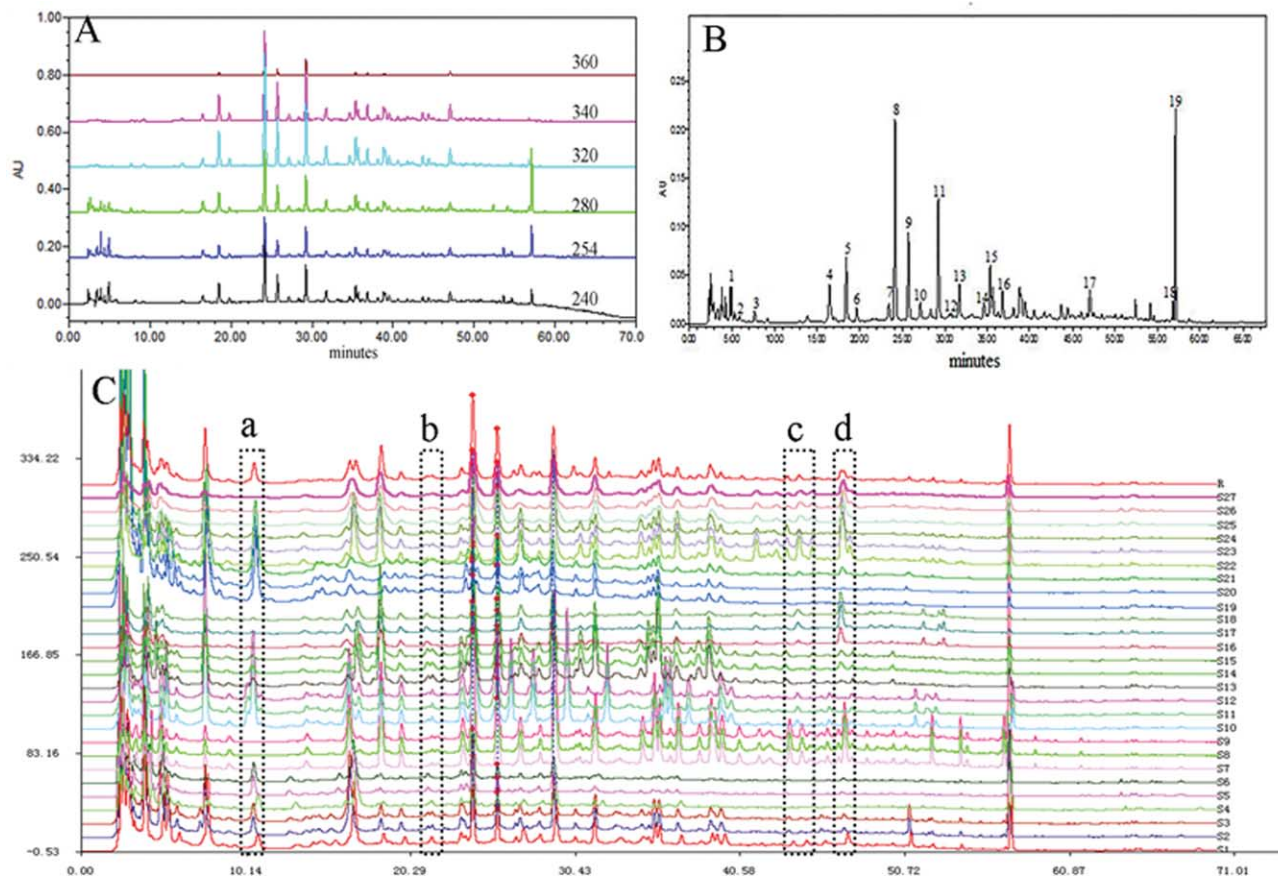
where  $X_i$  was the peak area in different fingerprints,  $Y_i$  was the mean peak area of twenty-seven samples and n was the number of peaks.

#### 6.3. Hierarchical cluster analysis (HCA) and principal component analysis (PCA)

HCA<sup>[29]</sup> is a multivariate analysis technique so as to find relatively homogeneous clusters in view of the characteristics of the cases. PCA<sup>[30–31]</sup> is applied to determine underlying information from multivariate raw data by transforming and reducing the dimensions of the original data matrix for n samples and m variables into a product of two matrices, scores (T) and loadings (P), while containing the same information as of the original data<sup>[32]</sup>. The information about the samples is presented in the form of scores, while loadings focus on the variables that have the most influence over the difference between groups of samples. This new, smaller set contains the uncorrelated components called principal components (PCs), the linear combinations of the original variables<sup>[33]</sup>. The two multivariate statistical methods confirm mutually and each approach possesses own advantage that HCA reveals patterns across whole cases while PCA across variables.

#### 6.4. Principal components regression (PCR) and partial least squares (PLS)

For the fingerprint-efficacy investigation, comparison of PCR and PLS was conducted to select preferable one to establish satisfactory calibration models<sup>[25]</sup>. PCR is a combination of PCA and least-squares regression<sup>[34]</sup>. Firstly PCA is performed to reduce the number of predictor variables as mentioned above. Some of these latent variables are used to build a predictive model by least-squares regression. PLS<sup>[35–36]</sup> expresses the inner relationship between independent variables (the m×n data matrix X) and dependent variables (y), which are simultaneously modeled by taking into account not only X variance, but the covariance between X and y. Generally, model complexity, such as the optimal number of PCs (latent variables) in PCR and latent factors in PLS, is estimated through the cross-validation procedure<sup>[37]</sup>. In order to characterize model suitably and prediction abilities, root mean square error (RMSE) and root mean square error of cross-validation (RMSECV) are usually calculated respectively<sup>[38]</sup>.



**Figure 2.** The comparison of fingerprint spectra in the different wavelengths (A). The common peaks (peaks 1-19, peak 8, 10 were chlorogenic acid and caffeic acid respectively) (B). The fingerprint chromatograms of 27 TMX samples at a wavelength of 280 nm (The chromatogram "R" was the standard fingerprint of 27 TMX samples) (C).

## RESULTS AND DISCUSSION

### 1. HPLC Fingerprints

Reversed-phase HPLC procedure was conducted in this work. Varying the ratios of 0.1% formic acid water and acetonitrile in the mobile phase provided a significant improvement in separation by yielding narrow and better-resolved peaks. The DAD wavelengths were monitored, Figure 2A showed the comparison of fingerprint spectra in the different wavelengths, and UV absorption at a wavelength of 280 nm was chosen because it generated the highest responses for the detection of all peaks. The HPLC fingerprints of 27 TMX samples at a wavelength of 280 nm were shown in Figure 2C. Twenty-eight peaks were labeled in the chromatographic fingerprints of 31 samples as input data for data analysis, if some peaks of the corresponding retention time were not found in the chromatogram of sample, the peak area was expressed used zero.

Nineteen peaks with large areas and good resolution under the present chromatographic conditions were collectively presented in chromatographic fingerprints of 27 TMX samples and selected as the common characteristic peaks (Figure 2B). Among of 19 common peaks, the 8th and 9th peak were identified as chlorogenic acid and caffeic acid by comparing with the chlorogenic acid standard and caffeic acid standard. Furthermore, the chlorogenic acid and caffeic

acid were at P13 and P15 in twenty-eight peaks of full spectrum respectively. The chlorogenic acid with the highest intensity in chromatography fingerprints of 27 TMX samples was chosen only as the reference peak to calculate the relative retention time (RRT) and relative peak area (RPA) for 19 common peaks in method validation.

### 2. Method Validation

To ensure the validity of this proposed method, the method precision was investigated by injecting sample 1 extracts on the same day and on three consecutive days for intra-day and inter-day variability with the selected condition. The variation of the nineteen common peaks was expressed as RSDs. The RSDs of intra-day variability were below 1% for RRT and 5% for RPA. For inter-day precision, the RSDs were less than 0.6% for RRT and 5% for RPA. The sample stability was determined by analyzing a single sample solution stored at room temperature for 0, 2, 4, 6, 10, 24, 48 and 72 h. The RSDs of stability test were less than 1% for RRT and 5% for RPA, indicating that TMX extraction solution was stable within 72 h. Meanwhile, six replicates of sample 9 were subjected to extraction, and HPLC determination according to the methods discussed in Section 2.3.1 for testing the method repeatability. The results revealed that RSDs of RRT were less than 2% and RSDs of RRA were less than 5%, so the methods of sample preparation and chromatography analysis

**Table 2.** The calculated similarity values for 27 samples.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
S1	1	0.981	0.601	0.515	0.518	0.497	0.728	0.735	0.735	0.693	0.698	0.698	0.477	0.495	0.473
S2	0.981	1	0.62	0.505	0.503	0.499	0.749	0.742	0.742	0.749	0.734	0.734	0.531	0.545	0.522
S3	0.601	0.62	1	0.826	0.829	0.848	0.624	0.626	0.626	0.636	0.636	0.636	0.638	0.65	0.613
S4	0.515	0.505	0.826	1	0.992	0.928	0.689	0.697	0.697	0.434	0.453	0.453	0.545	0.561	0.561
S5	0.518	0.503	0.829	0.992	1	0.926	0.692	0.701	0.701	0.446	0.473	0.473	0.551	0.568	0.567
S6	0.497	0.499	0.848	0.928	0.926	1	0.683	0.691	0.691	0.481	0.507	0.507	0.586	0.599	0.597
S7	0.728	0.749	0.624	0.689	0.692	0.683	1	0.985	0.985	0.685	0.647	0.647	0.721	0.743	0.757
S8	0.735	0.742	0.626	0.697	0.701	0.691	0.985	1	0.981	0.676	0.657	0.657	0.703	0.729	0.739
S9	0.735	0.742	0.626	0.697	0.701	0.691	0.985	0.981	1	0.676	0.657	0.657	0.703	0.729	0.739
S10	0.693	0.749	0.636	0.434	0.446	0.481	0.685	0.676	0.676	1	0.937	0.937	0.713	0.706	0.686
S11	0.698	0.734	0.636	0.453	0.473	0.507	0.647	0.657	0.657	0.937	1	0.976	0.68	0.676	0.649
S12	0.698	0.734	0.636	0.453	0.473	0.507	0.647	0.657	0.657	0.937	0.976	1	0.68	0.676	0.649
S13	0.477	0.531	0.638	0.545	0.551	0.586	0.721	0.703	0.703	0.713	0.68	0.68	1	0.972	0.988
S14	0.495	0.545	0.65	0.561	0.568	0.599	0.743	0.729	0.729	0.706	0.676	0.676	0.972	1	0.979
S15	0.473	0.522	0.613	0.561	0.567	0.597	0.757	0.739	0.739	0.686	0.649	0.649	0.988	0.979	1
S16	0.381	0.401	0.557	0.476	0.483	0.579	0.582	0.593	0.593	0.524	0.526	0.526	0.636	0.665	0.651
S17	0.377	0.391	0.514	0.49	0.483	0.564	0.629	0.638	0.638	0.491	0.501	0.501	0.653	0.677	0.678
S18	0.384	0.404	0.576	0.495	0.51	0.591	0.585	0.597	0.597	0.531	0.536	0.536	0.652	0.682	0.666
S19	0.512	0.535	0.557	0.303	0.32	0.346	0.295	0.293	0.293	0.641	0.754	0.754	0.447	0.478	0.376
S20	0.531	0.555	0.544	0.321	0.335	0.348	0.312	0.308	0.308	0.653	0.774	0.774	0.471	0.434	0.39
S21	0.514	0.539	0.516	0.301	0.318	0.333	0.28	0.277	0.277	0.61	0.752	0.752	0.408	0.393	0.335
S22	0.498	0.538	0.702	0.66	0.658	0.665	0.77	0.755	0.755	0.647	0.619	0.619	0.788	0.826	0.812
S23	0.539	0.585	0.792	0.666	0.668	0.683	0.736	0.729	0.729	0.749	0.72	0.72	0.819	0.845	0.824
S24	0.541	0.589	0.79	0.675	0.677	0.694	0.754	0.742	0.742	0.774	0.744	0.744	0.855	0.854	0.85
S25	0.749	0.767	0.556	0.542	0.545	0.556	0.9	0.889	0.889	0.747	0.704	0.704	0.82	0.819	0.836
S26	0.761	0.782	0.558	0.543	0.546	0.559	0.903	0.886	0.886	0.773	0.725	0.725	0.837	0.835	0.852
S27	0.673	0.683	0.525	0.55	0.55	0.556	0.897	0.889	0.889	0.68	0.641	0.641	0.8	0.81	0.826
R	0.751	0.784	0.78	0.697	0.705	0.723	0.889	0.886	0.886	0.86	0.859	0.859	0.878	0.889	0.876

had good repeatability. All validation tests proved the quality of the studied method.

### 3. Chemometric methods

#### 3.1. Similarity analysis

The standard chromatogram was shown in Figure 2C. Based on the standard fingerprint, the similarity values of twenty-seven fingerprints of TMX were presented in Table 2. The results showed that all the similarity values of each sample with the standard fingerprint of TMX were in a range of 0.575–0.913. The similarity of IR samples (S7–S9) with other IR samples (S22–S27) was higher than IH samples based on the high similarity values (0.729–0.9). It was clear that fingerprint characteristics of IH and IR from different geographical regions were difference.

#### 3.2. Hierarchical cluster analysis (HCA)

HCA was conducted through Average Linkage (Between Groups) as a clustering method and Euclidean Distance was used to measure distances between the cases and clusters<sup>[39]</sup>. A dendrogram was generated from HPLC files (Figure 3). The thirty-one samples were divided into three main clusters (I, II and III). Cluster I included eighteen IH samples (S1–S6, S10–S21). Cluster II consisted of nine IR samples (S7–S9, S22–S27). Four AL samples (S28–S31) were classified as cluster III. The results were in good agreement with that of plant morphological identification. Samples from the same source or region with the similar longitude and latitude could be gathered together well and the differences between

them were small. This fact demonstrated that the quality of the IH, IR and AL from different regions had significant difference.

In this work, the studied compounds were mostly the principle components of IH, IR and AL. Therefore, the IH and IR could be roughly compared from a distance of the clusters. The results of clustering analysis were consistent with similarity analysis.

#### 3.3. Principal component analysis (PCA)

PCA was processed by using peaks area of twenty-eight common peaks from 31 chromatograms as input data for calculation and all data were mean-centered. The first three principal components (PC 1, PC 2 and PC 3) describing 48%, 24% and 11% of the variability in the original observations respectively were extracted for analysis. The scatter plot of PCA scores was shown in Figure 4A, where each sample was represented as a dot. The plot described that the samples could be clearly classified into three domains. Samples 1–6 and 10–22 were in domain I, samples 7–9 and 23–27 were in domain II, while the others were in domain III. Domain I–III were marked for the IH, IR and AL respectively. Classification results were in line with clustering analysis results. The two pattern recognition analysis could provide a qualitative comparison and effectively distinguish the IH, IR and AL samples from different regions.

Since PC 1 and PC 2 explained 72% of the total variance, the loadings on these PCs (Figure 4B) were studied to interpret the contributions of the real variables to the model.

Table 2. Extended

S16	S17	S18	S19	S20	S21	S22	S23	S24	S25	S26	S27	R
0.381	0.377	0.384	0.512	0.531	0.514	0.498	0.539	0.541	0.749	0.761	0.673	0.751
0.401	0.391	0.404	0.535	0.555	0.539	0.538	0.585	0.589	0.767	0.782	0.683	0.784
0.557	0.514	0.576	0.557	0.544	0.516	0.702	0.792	0.79	0.556	0.558	0.525	0.78
0.476	0.49	0.495	0.303	0.321	0.301	0.66	0.666	0.675	0.542	0.543	0.55	0.697
0.483	0.483	0.51	0.32	0.335	0.318	0.658	0.668	0.677	0.545	0.546	0.55	0.705
0.579	0.564	0.591	0.346	0.348	0.333	0.665	0.683	0.694	0.556	0.559	0.556	0.723
0.582	0.629	0.585	0.295	0.312	0.28	0.77	0.736	0.754	0.9	0.903	0.897	0.889
0.593	0.638	0.597	0.293	0.308	0.277	0.755	0.729	0.742	0.889	0.886	0.889	0.886
0.593	0.638	0.597	0.293	0.308	0.277	0.755	0.729	0.742	0.889	0.886	0.889	0.886
0.524	0.491	0.531	0.641	0.653	0.61	0.647	0.749	0.774	0.747	0.773	0.68	0.86
0.526	0.501	0.536	0.754	0.774	0.752	0.619	0.72	0.744	0.704	0.725	0.641	0.859
0.526	0.501	0.536	0.754	0.774	0.752	0.619	0.72	0.744	0.704	0.725	0.641	0.859
0.636	0.653	0.652	0.447	0.471	0.408	0.788	0.819	0.855	0.82	0.837	0.8	0.878
0.665	0.677	0.682	0.478	0.434	0.393	0.826	0.845	0.854	0.819	0.835	0.81	0.889
0.651	0.678	0.666	0.376	0.39	0.335	0.812	0.824	0.85	0.836	0.852	0.826	0.876
1	0.931	0.976	0.298	0.309	0.344	0.843	0.833	0.795	0.616	0.601	0.622	0.715
0.931	1	0.885	0.229	0.246	0.259	0.837	0.804	0.803	0.645	0.655	0.674	0.716
0.976	0.885	1	0.313	0.324	0.361	0.86	0.852	0.803	0.629	0.606	0.634	0.728
0.298	0.229	0.313	1	0.899	0.881	0.336	0.465	0.469	0.364	0.38	0.292	0.588
0.309	0.246	0.324	0.899	1	0.966	0.35	0.477	0.486	0.399	0.415	0.324	0.605
0.344	0.259	0.361	0.881	0.966	1	0.374	0.487	0.464	0.35	0.365	0.268	0.575
0.843	0.837	0.86	0.336	0.35	0.374	1	0.97	0.931	0.763	0.756	0.784	0.866
0.833	0.804	0.852	0.465	0.477	0.487	0.97	1	0.97	0.748	0.749	0.744	0.902
0.795	0.803	0.803	0.469	0.486	0.464	0.931	0.97	1	0.764	0.782	0.749	0.913
0.616	0.645	0.629	0.364	0.399	0.35	0.763	0.748	0.764	1	0.985	0.973	0.905
0.601	0.655	0.606	0.38	0.415	0.365	0.756	0.749	0.782	0.985	1	0.957	0.914
0.622	0.674	0.634	0.292	0.324	0.268	0.784	0.744	0.749	0.973	0.957	1	0.875
0.715	0.716	0.728	0.588	0.605	0.575	0.866	0.902	0.913	0.905	0.914	0.875	1

The weight values of the important variables (P7, P9, P22, P24, P26 and P11) were 0.984, 0.983, 0.977, 0.932, 0.908 and 0.906 respectively, these six characteristic peaks had a primary contribution to distinguish samples. Also, we could find different peaks from the Figure 2C. Region a: P7 (retention time 10.236) didn't exist in the fingerprints of all IR samples. Region b: there was no P11 (retention time 22.22) in nine IH samples (S13–S21). Region c: other samples had the P26 (retention time 42.362), besides S4–S6 and S18–S20. Region d: only three IH samples (S4–S6) didn't detect the P27 (retention time 47.048).

#### 4. Anti-inflammatory activity

The aqueous extract of 27 TMX samples (9 g/kg) was found to significantly (Figure 5;  $P < 0.01$ ) inhibit auricle edema formation when compared to the blank group. S7 appeared the highest anti-inflammatory capacity ( $P < 0.05$ ) compared with six IH samples (S4–S6, S19–S21) and six IR samples (S22–S27).

### 5. Spectrum-efficacy relationship

#### 5.1. Principal component regression (PCR)

Three PCs (1, 2 and 3) were selected to build a PCR model from the chromatograms in view of their ability to predict  $y$  assessed by full cross-validation when RMSE and RMSECV were 0.1357 and 0.1278 respectively. Figure 6A showed that the P2, P5, P16, P17, P20 and P27 appeared significant negative contributions to anti-inflammatory activities, and the contribution ratio of the 9 characteristic peaks that had

greater contribution to anti-inflammatory capacity ranked as  $P15 > P11 > P19 > P6 > P1 > P18 > P12 > P22 > P24$ . Although it was known that P13 (chlorogenic acid) possessed strong anti-inflammatory activities<sup>[40]</sup>, it should have contribute to the model, yet this peak displayed suppression anti-inflammatory effect by PCR. This may be caused by the fact that PCR is primarily searching for the largest variation in the data, which is not necessarily the information in the chromatogram most correlated with the anti-inflammatory capacity<sup>[41]</sup>.

#### 5.2. Partial least squares (PLS)

The PLS was also employed to establish spectrum-efficacy model. The auricle edema rate was chosen as the dependent variable  $y$  and the whole data set of fingerprints after preprocessing as the independent variables  $X$ . The PLS model with two factors was retained for auricle edema rate with chromatograms by full cross-validation.

Figure 6B illustrated that the P2, P5, P20, P27 appeared greater negative contributions to anti-inflammatory activities, most of peaks appeared positive contributions to the anti-inflammatory activities and the magnitude of the 10 characteristic peaks that had close correlation with anti-inflammatory activities was:  $P15 > P19 > P6 > P11 > P18 > P12 > P10 > P22 > P24 > P13$ . The outcome nearly coincided with the results in PCR. Contrary to the result in PCR, the regression coefficient of P13 was correctly positive in the PLS model. Consequently, the interpretation and accuracy of the model established by PLS were described more clearly than

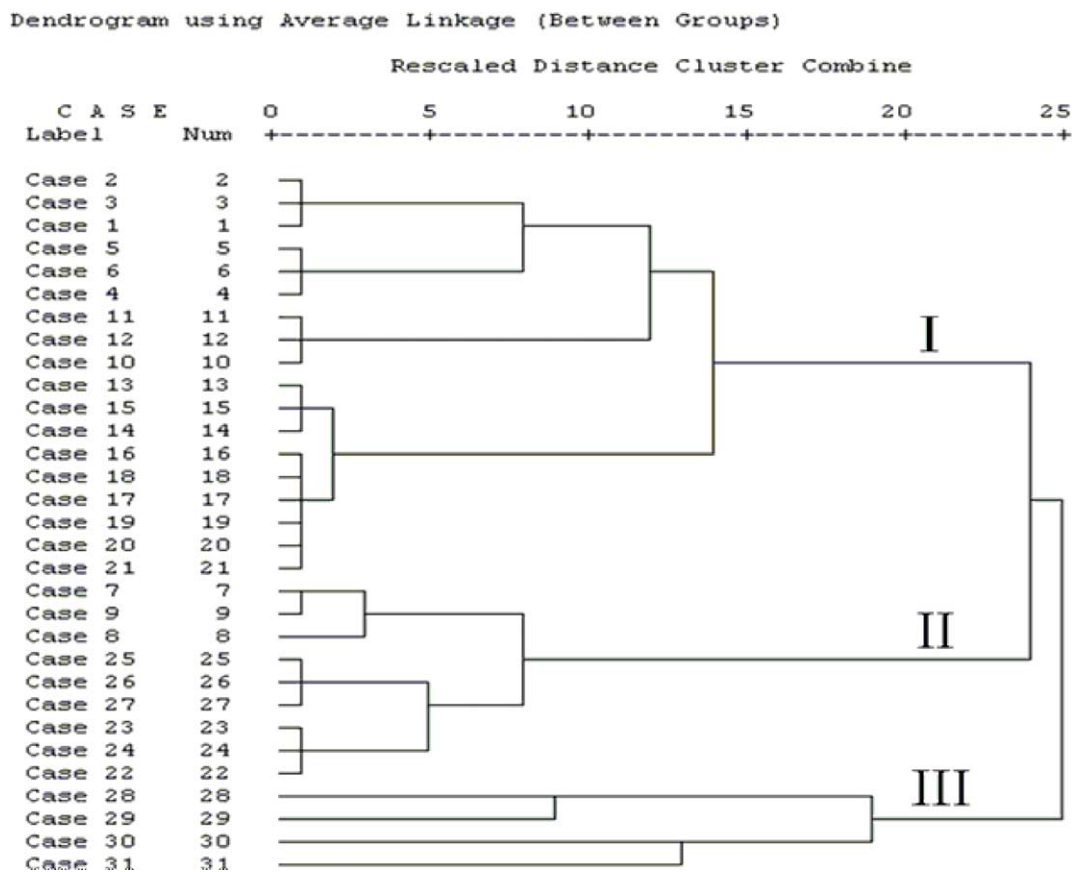


Figure 3. Hierarchical clustering dendrogram of the samples.

PCR. An advantage of PLS is that this technique not only looks for maximal variance in X (the chromatographic data) as PCR, but also considers the correlation between the chromatographic data (X) and the total capacity (y), which results in lower RMSE (0.1133) and RMSECV (0.1084) values than PCR.

Overall, both PCR and PLS could be used to simultaneously screen the main contributive peaks to the anti-inflammatory capability of TMX. PLS provided a more accurate spectrum-activity model than PCR revealed that the 10 characteristic peaks that had most closely correlation with anti-inflammatory capacity.

The anti-inflammatory effects of different samples also could be explained by the results of spectrum-efficacy

relationship. For example, 4 characteristic peaks (P6, P11, P15 and P22) which appeared positive contributions to anti-inflammatory capacity were not found in the fingerprints of samples 19-21, therefore, these samples had lowest anti-inflammatory activities. Also, the P22 didn't exist in IR samples of Gannan and Tibet, so the anti-inflammatory activities of these samples were relatively low.

### 6. HPLC-MS-MS analysis of TMX

The TIC chromatograms of S7 by HPLC-MS-MS in negative and positive ESI mode were shown in Figure 7A. The constituents of TMX were well separated and identified by using their retention time and mass spectra. By comparing

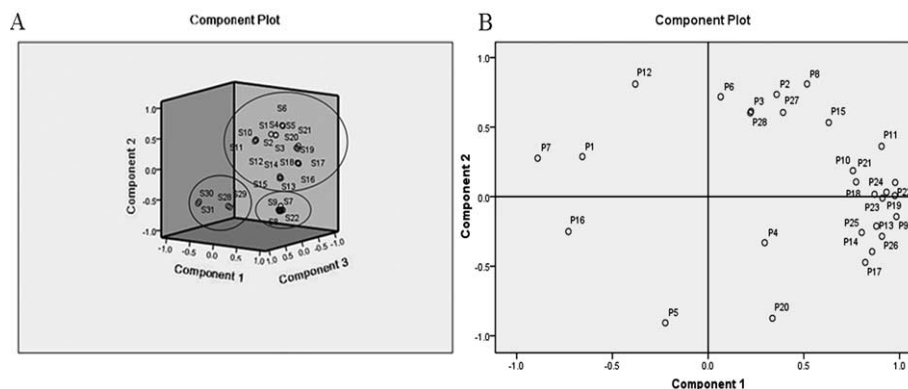
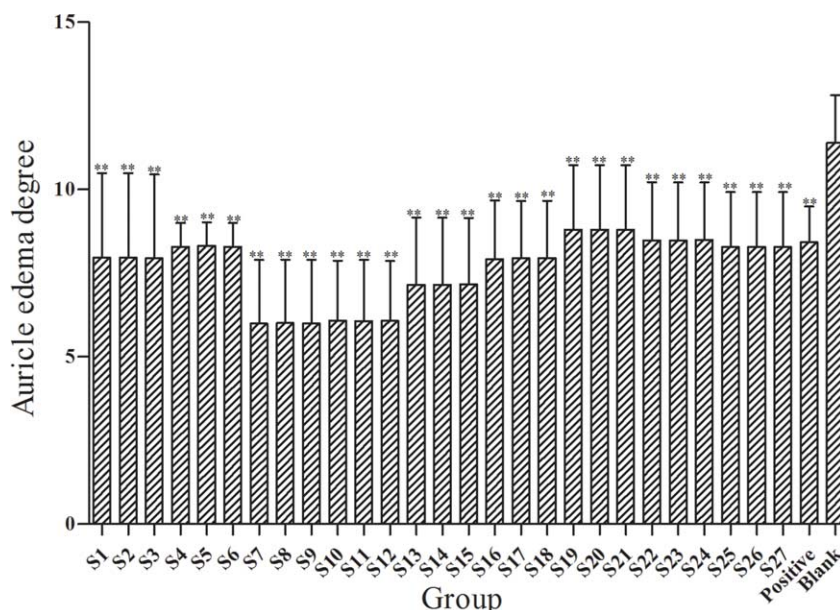


Figure 4. The score plot for thirty-one samples (A) and corresponding loading plot (B).





**Figure 5.** The anti-inflammatory effects of 27 samples. Significant differences with blank group are designated as \*\* $P < 0.01$ .

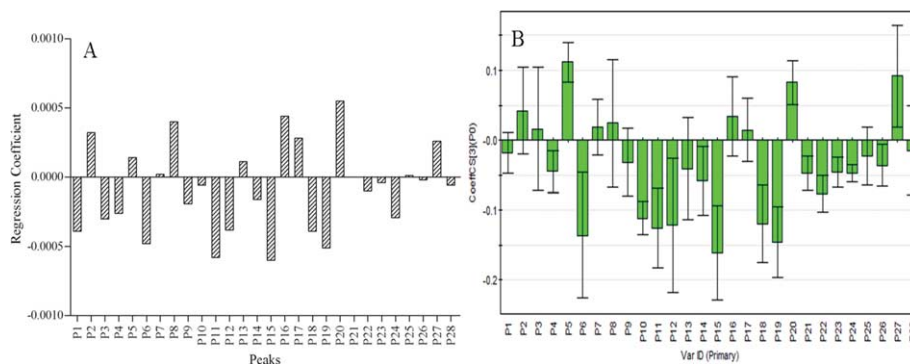
with their resolved spectra with the standard ones, 8 characteristic peaks which had close correlation with anti-inflammatory activities were identified. As a result, peak 6, 11, 12, 13, 15, 18, 22, 24 were identified as citric acid, quinic acid, caffeic acid- $\beta$ -D-glucopyranoside, chlorogenic acid, caffeic acid, 1,3-O-dicaffeoyl quinic acid, tianshic acid and 3 $\beta$ -Hydroxypterondontic acid respectively. The chemical structures, mass spectra and possible fragmentation pathway of these 8 peaks were shown in Figure 7B. The anti-inflammatory activities of citric acid<sup>[42]</sup>, quinic acid<sup>[43]</sup> and caffeic acid<sup>[44]</sup> were also reported in the literature.

## CONCLUSIONS

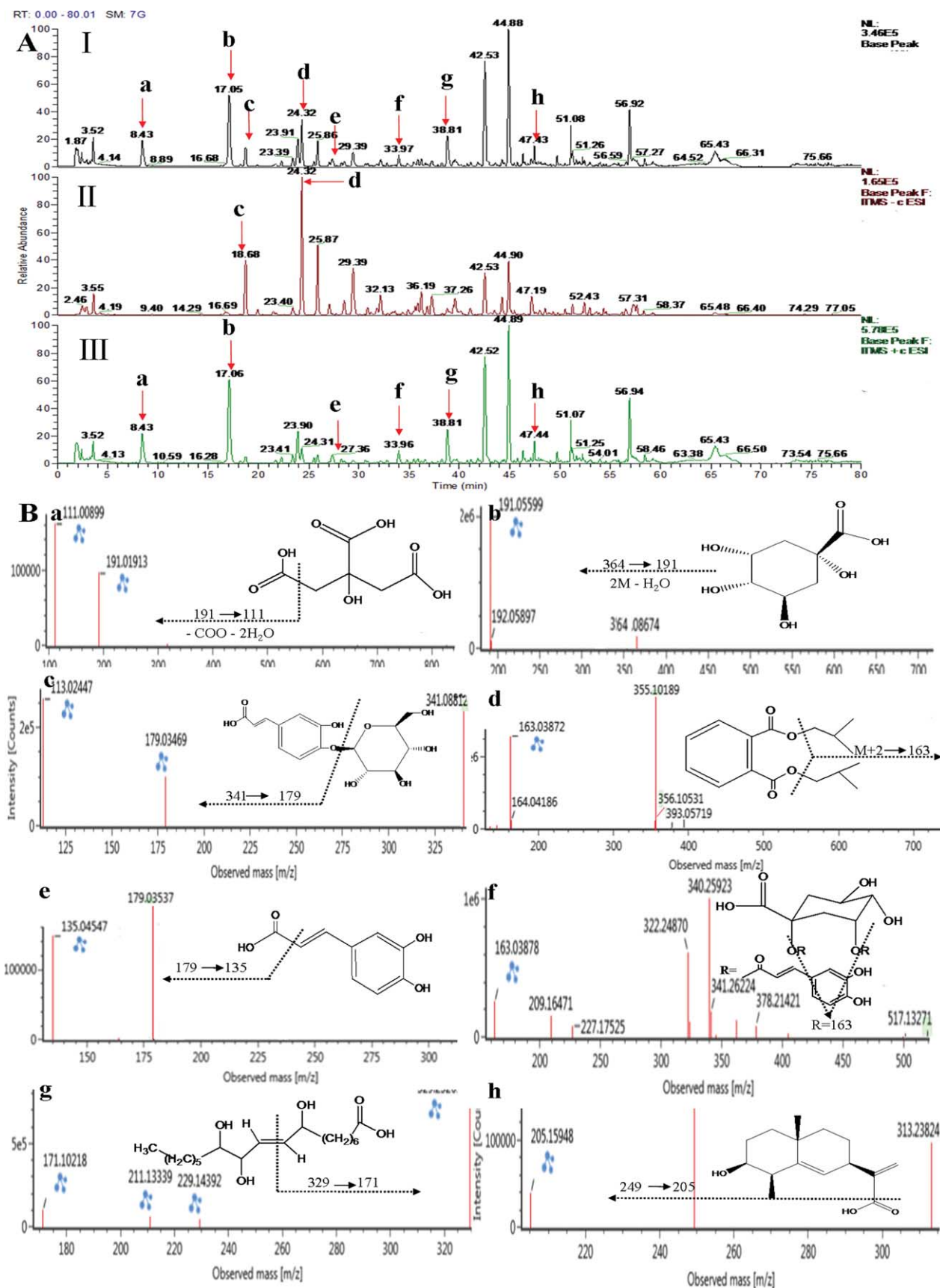
In conclusion, a holistic method based on chemical characteristic and anti-inflammation effect was established to systematically evaluate the quality of twenty-seven TMX samples from different origins. An integrative outcome revealed that all samples were successfully classified. The results were in accord with the appraisal results of plant morphological. The results of the anti-inflammatory activities

showed that S7 from Kumbum Monastery of Qinghai exhibited significant anti-inflammatory capacity. Meanwhile, citric acid, quinic acid, caffeic acid- $\beta$ -D-glucopyranoside, chlorogenic acid, caffeic acid, 1,3-O-dicaffeoyl quinic acid, tianshic acid and 3 $\beta$ -Hydroxypterondontic acid had significant positive correlation with anti-inflammatory activities by means of investigating spectrum-efficacy relationship, most of these active ingredients were phenolic acid compounds. These results were consistent with the reports that both of IH and IR contained abundant phenolic acid compounds<sup>[45]</sup>, and phenolic acids compounds and their derivatives processed superior anti-inflammatory activities<sup>[46]</sup>.

TCMs are mostly used in the clinic with aqueous extract. Alantolactone and isoalantolactone were not found in the aqueous extract of TMX. However, these two active ingredients were selected as chemical markers for the quality control of TMX in ChP 2015<sup>[3]</sup>. Thus, alantolactone and isoalantolactone may not be appropriate as the markers for the quality control of TMX associated with anti-inflammatory activities. According to our research, it is suggested that phenolic acid compounds and their derivatives can be selected



**Figure 6.** The regression coefficients of PCR (A) and regression coefficient figure of PLS (B).



**Figure 7.** The base peak chromatogram of S7(A,I); The total ion chromatograms (TIC) of S7 in negative ion mode (A,II); The total ion chromatograms (TIC) of S7 in positive ion mode (A, III); Chemical structures, mass spectra and possible fragmentation pathway of the compounds (B) (a, citric acid; b, quinic acid; c, caffeic acid-β-D-glucopyranoside; d, chlorogenic acid; e, caffeic acid; f, 1,3-O-dicaffeoyl quinic acid; g, tianshic acid; h, 3β-Hydroxypterondontic acid).

as the chemical markers for anti-inflammatory relevant quality assessment of TMX. Consequently, we should pay close attention to the variation of citric acid, quinic acid, caffeic acid- $\beta$ -D-glucopyranoside, chlorogenic acid, caffeic acid, 1,3-O-dicaffeoyl quinic acid, tianshich acid and 3 $\beta$ -Hydroxypterondontic acid in the future quality control study of TMX associated with anti-inflammatory effect.

This comprehensive strategy was successfully used for identification of IH, IR and AL, which provided a reliable and adequate theoretical basis for formulating the bioactivity relevant quality standards and studying the material basis of anti-inflammatory effect of TMX, and provided a methodology reference for quality evaluation and analyzing related pharmacodynamic material basis of other TCMs.

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